Malolactic Fermentation: The ABC's of MLF

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There are two main fermentations associated with the winemaking process. Alcoholic fermentation is conducted by the yeast culture and malolactic fermentation takes place as a result of the metabolic activity of lactic acid bacteria, specifically from the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc*. Malolactic fermentation is defined as the conversion of malic acid to lactic acid and CO_2 and besides deacidification also contributes to microbial stability and modification of the aroma profile. This paper aims to provide a comprehensive review discussing all the main aspects and factors related to malolactic fermentation, including practical considerations for monitoring and ensuring a successful fermentation.

INTRODUCTION

Malolactic fermentation (MLF) is an intricate process that usually follows after the completion of alcoholic fermentation (AF) by yeasts. Although MLF is regarded as a secondary fermentation process, it plays an integral role in the production of the majority of red wines, as well as some white cultivars including Chardonnay and some sparkling wines. There are three main reasons for conducting MLF in wine. Firstly, the deacidification of the wine with a concomitant increase in pH, secondly, to contribute to the microbial stability by the removal of malic acid as a possible carbon substrate and thirdly, the modification of the wine aroma profile (Davis *et al.,* 1988; Kunkee, 1991; Maicas *et al.*, 1999; Liu, 2002; Ugliano *et al.,* 2003). In cooler climate countries the deacidification process is regarded as the most important modification associated with MLF, while the change in the sensory profile of the wine is a more important consideration in countries where deacidification is of less significance, i.e. warmer regions where lower concentrations of malic acid are present in the grapes.

The MLF reaction is defined as the conversion of L-malic acid, a dicarboxylic acid, to L-lactic acid, a monocarboxylic acid, with the production of CO_2. The reaction is catalysed by lactic acid bacteria (LAB), including bacteria from the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Wibowo *et al.,* 1985). Of these, *Oenococcus oeni* is best adapted to the harsh wine environment, including conditions of high alcohol, low pH and the presence of sulphur dioxide (SO_2) (Wibowo *et al.*, 1985; Davis *et al.,* 1988; Drici-Cachon *et al.,* 1996; Lonvaud-Funel, 1999). Various review articles on MLF have appeared over the years (Wibowo *et al.*, 1985; Davis *et al.,* 1988; Kunkee, 1991), with increasing amounts of information being generated regarding this important step in the winemaking process as well as the characterisation of the microorganisms involved. Some of the most recent review articles include Lonvaud-Funel (1999), Liu (2002) and Bauer & Dicks (2004), with the focus falling on the metabolism of wine associated LAB, specifically *O. oeni,* and factors influencing LAB and MLF. In addition to the information being generated on the metabolic processes associated with wine LAB, the molecular aspects of LAB are also being investigated. At the beginning of the 21st century, the DOE Joint Genome Institute commenced the sequencing of the entire genome of *O. oeni* PSU-1, a strain isolated by Beelman and co-workers (Bartowsky, 2005). The genome is now fully sequenced, which allows for more intensive studies regarding the physiology, genetic diversity and performance of *O. oeni* starter cultures.

The focus of this literature review will be to summarise key aspects associated with the process of MLF. The MLF reaction as well as the main LAB found in wine will be discussed. The use of commercial starter cultures and the influence of different inoculation times are considered. Various factors influence this fermentation process, such as wine parameters, microorganisms and compounds originating from the grapes, and will also be discussed. As recent research focus has fallen on the organoleptic changes in wine undergoing MLF, the important aroma compounds responsible for MLF aroma characteristics are critically reviewed. The final section of the review will highlight some practical considerations for the monitoring of MLF to ensure the successful completion of MLF with a positive contribution to the aroma profile.

MALOLACTIC FERMENTATION

Lactic acid bacteria possess three possible enzymatic pathways for the conversion of L-malic acid to L-lactic acid and CO_2. The first is the direct conversion of malic acid to lactic acid via malate decarboxylase, also known as the malolactic enzyme (MLE). This reaction requires NAD^+ and Mn^{2+} as cofactors and no free intermediates are produced during this decarboxylation reaction. The rate of malate decarboxylation by LAB is correlated to the specific malolactic activity of the bacterial cell (Bartowsky, 2005). The main wine LAB utilise this pathway to generate lactic acid. A paper written by Lonvaud-Funel (1995) highlighted the main features of the malate decarboxylase (*mleA*) gene. The enzyme has been purified from various LAB species that were isolated from wines and grapes, including species from *Lactobacillus* and

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Leuconostoc (Lonvaud-Funel, 1995). The second pathway utilises the malic enzyme to convert L-malic acid to pyruvic acid, which is subsequently reduced by L-lactate dehydrogenase to lactic acid. The third possible pathway is the reduction of malate by malate dehydrogenase to oxaloacetate, followed by decarboxylation to pyruvate and reduction to lactic acid (Lonvaud-Funel, 1999).

The major physiological function of the malate fermentation pathway is to generate a proton motive force (PMF) as a means to acquire energy to drive essential cellular processes (Konings, 2002). The MLF reaction catalysed by the MLE enzyme can be divided into three stages: the uptake of L-malic acid by wine LAB, the decarboxylation of L-malic acid to L-lactic acid and CO_2 and the excretion of L-lactic acid together with a proton. The decarboxylation reaction yields an electrical potential ($\Delta\psi$). The proton that is secreted during the decarboxylation reaction results in an increase in the internal pH of the bacterial cell which yields a pH gradient (Δ_{pH}) across the membrane. These two components make up the PMF which then generate ATP via membrane ATPases. The PMF is sufficient to drive energy-consuming reactions e.g. the transport of metabolites (Henick-Kling, 1993; Versari *et al.*, 1999).

LACTIC ACID BACTERIA ASSOCIATED WITH WINE

Lactic acid bacteria are coccoid to elongated cocci or rod-shaped bacilli, Gram-positive, non-sporing and non-respiring bacteria. As the name suggests, lactic acid is the major product formed during the fermentation of carbohydrates. Lactic acid bacteria species from the genera *Leuconostoc, Pediococcus, Lactobacillus* as well as *O. oeni*, are accountable for the changes to the wine matrix during the fermentation process (Wibowo *et al.,* 1985). *Oenococcus oeni* has best adapted to the wine environment and concomitantly the majority of LAB present in wine belong to this species. *Oenococcus oeni* strains are also the selected bacteria used for commercial starter cultures (Wibowo *et al.*, 1985; Davis *et al.,* 1988; Drici-Cachon *et al.,* 1996; Lonvaud-Funel, 1999).

Evolution of the lactic acid bacteria population

The evolution of LAB from the vineyard to the final vinification stages have been documented, but show considerable variability due to region, cultivar and vinification procedures. It is clear that there is a successional growth of several species of LAB during vinification (Wibowo *et al.*, 1985; Boulton *et al.*, 1996; Fugelsang & Edwards, 1997). *Oenococcus oeni* is the main LAB species associated with wine; *Pediococcus damnosus*, *Pediococcus parvulus* and *Pediococcus pentosaceus* mostly occur after MLF and in higher pH wines and several *Lactobacillus* species also occur after MLF (Wibowo *et al.*, 1985; Powell *et al.*, 2006).

In the vineyard, the diversity and population density of LAB are very limited, especially in comparison to the indigenous yeast population found on grapes (Fugelsang & Edwards, 1997). Organisms occur on grapes and leaf surfaces (Wibowo *et al.*, 1985) but population numbers on undamaged grapes and grape must are rarely higher than 10^3 CFU/g (Lafon-Lafourcade *et al.*, 1983). The population size on grape surfaces depend in large on the maturity and sanitary state of the grapes (Wibowo *et al.*, 1985; Jackson, 2008) and *Pediococcus* and *Leuconostoc* species occur on grapes more frequently than *O. oeni* (Jackson, 2008). Besides grape surfaces, bacterial strains can also be isolated from the cellar environment, including barrels and poorly sanitised winery equipment like pipes and valves (Donnelly, 1977; Boulton *et al.*, 1996; Jackson, 2008).

Shortly after crushing and the start of AF, the LAB population in the grape must generally range from 10^3 to 10^4 CFU/mL. The major species of LAB present at this stage include *Lactobacillus plantarum*, *Lactobacillus casei*, *Leuconostoc mesenteroides*, and *P. damnosus,* as well as *O. oeni* to a lesser extent (Wibowo *et al.*, 1985; Lonvaud-Funel *et al.*, 1991; Boulton *et al.*, 1996; Powell *et al.*, 2006). Most of these LAB species generally do not multiply and decline towards the end of AF, with the exception of *O. oeni* (Wibowo *et al.*, 1985; Lonvaud-Funel *et al.*, 1991; Van Vuuren & Dicks, 1993; Fugelsang & Edwards, 1997; Volschenk *et al.*, 2006). The decrease could be attributed to increased ethanol concentrations, high SO_2 concentrations, low pH, low temperatures, the nutritional status and competitive interactions with the yeast culture (Fugelsang & Edwards, 1997; Volschenk *et al.*, 2006).

After the completion of AF and the bacterial lag phase, the surviving bacterial cells, most commonly *O. oeni*, start to multiply. This phase is characterised by vigorous bacterial growth and the start of MLF is induced when bacterial populations reach 10^6 CFU/mL (Wibowo *et al.*, 1985; Lonvaud-Funel, 1999). The pH of the wine is imperative in determining which species of LAB are present, with values above pH 3.5 favouring the growth of *Lactobacillus* and *Pediococcus* species, whereas the *O. oeni* population tend to dominate at lower pH values (Davis *et al.*, 1986b; Henick-Kling, 1993).

When MLF is complete, the remaining LAB are still able to metabolise residual sugar, which could result in spoilage including volatile acidity (Fugelsang & Edwards, 1997). This is particularly prevalent in high pH wines, where *Lactobacillus* and *Pediococcus* may occur and contribute to wine spoilage (Wibowo *et al.*, 1985). It is therefore imperative to control the potential impact of residual LAB populations after the completion of MLF to reduce the risk of spoilage.

By understanding the evolution of LAB from the vineyard/ grape surfaces, through the different vinification procedures, as well as their metabolic requirements, it is possible to control which species of LAB occur at a particular stage and to ensure that they make a positive contribution during MLF.

Metabolism of lactic acid bacteria

Metabolism of carbohydrates

Lactic acid bacteria possess two main pathways for the metabolism of glucose and a single pathway for the metabolism of pentose sugars. The two pathways for the metabolism of glucose include the glycolysis/Embden-Meyerhof-Parnas (EMP) pathway and the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway (also referred to as the phosphoketolase- or pentose phosphate pathway) (Fugelsang & Edwards, 1997).

Glucose, as a free sugar, is transported into the cell where it is phosphorylated by hexokinase, a reaction which is ATP-dependant, before it enters one of the two mentioned pathways. The EMP pathway, also known as homolactic fermentation in LAB, leads to the formation of lactic acid as the main end-product, as well as the production of CO_2. This pathway is divided into two steps. The first reaction is glycolysis, whereby pyruvate is produced from glucose, followed by the conversion of pyruvate to

produce lactic acid (Ribéreau-Gayon *et al.*, 2006). This pathway is utilised by *Pediococcus* strains and the metabolism of one mole of glucose produces two moles of lactic acid as well as a net amount of two ATP. The 6-PG/PK pathway, also known as heterolactic fermentation, results in the production of lactic acid and CO_2, as well as the end-products ethanol and acetate. Species of LAB that make use of this pathway include all the strains of *Leuconostoc*, some *Lactobacillus* strains and *O. oeni*. One mole of glucose metabolised via this pathway will lead to the formation of equimolar amounts of each of lactic acid, ethanol and CO_2, as well as one mole of ATP (Fugelsang & Edwards, 1997).

Many LAB are able to ferment pentose sugars and special permeases are used for entry of pentose sugar into the cell. Pentoses are phosphorylated, converted by epimerases or isomerases to phosphate derivatives ribulose-5-phosphate or xylulose-5-phosphate, after which they are metabolised via the bottom half of the 6-PG/PK pathway. The end-products of pentoses metabolism are equimolar amounts of lactic acid, acetic acid and CO_2.

According to the pathway used for the metabolism of carbohydrates, LAB can be divided into three metabolic groups. Each group also differs according to the enzymes that are needed for carbohydrate metabolism. The obligatory homofermentors only make use of the EMP pathway for carbohydrate metabolism. They possess the aldolase enzyme but the phosphoketolase enzyme is absent. All wine *Pediococcus* species are included in this group. The obligatory heterofermentors include *Lactobacillus brevis*, *Lactobacillus hilgardii*, *Leuconostoc* species and *O. oeni*. This group utilises the 6-PG/PK fermentation pathway for the metabolism of carbohydrates. This group displays phosphoketolase activity but do not possess the aldolase enzyme. Some *Lactobacillus* species are facultative heterofermentors. These include *L. casei* and *L. plantarum*. These LAB make use of the EMP pathway for hexose metabolism and the 6-PG/PK pathway for the metabolism of pentose sugars and other substrates. These LAB only possess the aldolase enzyme (Fugelsang & Edwards, 1997).

An understanding of the metabolic requirements of LAB will aid the winemaker in making decisions regarding the nutrient requirements and management during MLF.

INDUCTION OF MALOLACTIC FERMENTATION

Commercial starter cultures

Winemakers are starting to recognise the benefits of inoculating grape must or wine with commercial starter cultures of LAB to ensure the successful completion of MLF (Davis *et al.*, 1985; Fugelsang & Zoecklein, 1993; Henick-Kling, 1995; Krieger-Weber, 2009) and to reduce the risks associated with spontaneous MLF. Potential risks include the presence of unidentified/spoilage bacteria that can produce undesirable or off-flavours, the production of biogenic amines (Davis *et al.*, 1985), a delay in the onset or completion of MLF (Nielsen *et al.*, 1996) and the development of bacteriophages (Bauer & Dicks, 2004); all of which contribute to a decrease in the quality of the wine (Bartowsky & Henschke, 1995; Fugelsang & Edwards, 1997). By inoculating with a commercial starter culture, most of which contain *O. oeni* as the single LAB culture, the winemaker can reduce the risk of potential spoilage bacteria or bacteriophages, promote the rapid start and completion of MLF and also encourage a positive flavour contribution by the LAB (Krieger-Weber, 2009). Recently, *L. plantarum* has also been considered for application in a commercial starter culture (Bou & Krieger, 2004).

Lactic acid bacteria strain ML34 served as the prototype in the 1960's to 1970's for the development of the concept of inoculating for MLF with a single strain. Malolactic fermentation starter cultures were available in liquid form and used for decades until the early 1980's. At that time, frozen and freeze-dried LAB starter cultures were developed. Shortly after, in the 1990's, direct inoculation freeze-dried starter cultures were developed, with Viniflora oenos being the first (Nielsen *et al.*, 1996). Their use has made it easier to control and predict the progression of MLF in wine (Specht, 2006). These commercial cultures are also easy to ship, store and use, which adds to their increasing popularity. A commercial starter culture contains a very high population of viable bacteria, $\pm 10^{11}$ CFU/g, to ensure that any loss in viability due to the wine conditions is not detrimental to the completion of MLF (Henick-Kling, 1993, 1995). Table 1 gives a general overview of some of the commercial MLF starter cultures that are available at present.

There are various types or forms of LAB starter cultures available. The liquid suspension culture only has a shelf life of 2 to 20 days and requires a preparation time of 3 to 7 days. The frozen cultures need to be inoculated immediately after being thawed and the pellets are directly added to the wine. To the contrary, the direct inoculation (MBR®) culture does not need any special preparation and is directly added to the wine.

The quick build-up starter culture (1-STEP®) requires an additional activation step whereby an activator and wine is added to the culture 18 to 24 hours prior to inoculation in the wine. The traditional freeze-dried culture has to be rehydrated in a wine/water mixture and addition of the culture to the wine takes place over a period of 3 to 14 days.

In an effort to be more cost-effective, a technique referred to as stretching can be implemented. The stretching of starter cultures imply using less than the recommended dosage, but can also imply the re-use of commercial starter cultures as in the case of mother tank inoculation as well as inoculation from the lees of wines that have finished MLF. These are risky practices. There is a possibility of the development of spoilage microorganisms due to the decreased population of inoculated bacteria and MLF may not be successfully completed. Further risks include a lack of control over the MLF process as well as the contamination of further fermentation vessels from a contaminated mother tank (Van der Merwe, 2007). Due to the risks associated with spontaneous or uncontrolled MLF and stretching, it is important for the winemaker to realise the benefits associated with inoculating for MLF with a starter culture as well as inoculating according to the directions of the manufacturer.

The selection and characterisation of strains for possible use in a commercial culture is crucial, due to the fact that LAB strains differ in their fermentation capabilities and growth characteristics (Britz & Tracey, 1990; Henick-Kling, 1993). Strict criteria are used for the selection of bacteria to be used as starter cultures (Davis *et al.*, 1985; Vaillant *et al.*, 1995; Volschenk *et al.*, 2006; Krieger-Weber, 2009). These criteria include the following: tolerance to low pH, high ethanol and SO_2 concentrations, good growth characteristics under winemaking conditions, compatibility with

TABLE 1

A general overview of some of the MLF starter cultures that are available as well as their main characteristics and applications (compiled from company websites).

Name	Company	Characteristics	Application	Form
Viniflora CH16	Chr. Hansen	Temperature: 17-25°C Alcohol tolerance:16%.pH: 3.4 TSO_2* tolerance: 40 ppm	High alcohol red and some types of rosé wines	Frozen/Freeze-dried
Viniflora CH35	Chr. Hansen	Temperature: 15-25°C Alcohol tolerance: 14%. pH: 3.1 TSO_2 tolerance: 45 ppm	White and some rosé wines	Frozen/Freeze-dried
Viniflora CH11	Chr. Hansen	Temperature: 14-25°C Alcohol tolerance: 15%. pH: 3 TSO_2 tolerance: 35 ppm	Low pH white and some rosé wines	Frozen/Freeze-dried
Viniflora oenos	Chr. Hansen	Temperature: 17-25°C Alcohol tolerance: 14%. pH: 3.2 TSO_2 tolerance: 40 ppm	Red, rosé and white wines	Frozen/Freeze-dried
Viniflora Ciné	Chr. Hansen	Temperature: 17-25°C Alcohol tolerance: 14%. pH: 3.2 TSO_2 tolerance: 30 ppm	Red, rosé and white wines, sparkling wine with no diacetyl production	Frozen
Biolact Acclimatée	AEB Group	NA**	NA	Freeze-dried
Biolact Acclimatée BM	AEB Group	Temperature: 12°C. pH: 3	NA	Freeze-dried
Biolact Acclimatée PB1025	AEB Group	Temperature: 15-18°C Alcohol tolerance: high. pH: 2.9 TSO_2 tolerance: high	White, rosé and young red wines	Freeze-dried
Biolact Acclimatée 4R	AEB Group	Temperature: resistance to low temp. Alcohol tolerance: high	Red wines with high tannin concentrations	Freeze-dried
Lactoenos B16 Standard	Laffort	Temperature: >16°C Alcohol tolerance: 16% pH: >2.9 TSO_2 tolerance: 60 ppm	Acidic white wines	NA
Lactoenos SB3 Instant	Laffort	Temperature: >16°C Alcohol tolerance: 15%. pH: >3.3 TSO_2 tolerance: 30 ppm	High quality wines (undergoing barrel MLF)	NA
Lactoenos 350 PreAc	Laffort	Temperature: >15°C Alcohol tolerance: 16%. pH: >3 TSO_2 tolerance: 60 ppm	Low pH white and certain rosé wines	NA
Lactoenos 450 PreAc	Laffort	Temperature: >16°C Alcohol tolerance: 17%. pH: >3.3 TSO_2 tolerance: 80 ppm	Red and white wines	NA
1 Step Alpha	Lallemand	Temperature: 14°C Alcohol tolerance: high. pH: > 3.3 TSO_2 tolerance: < 40 ppm	Red and white wines	Freeze-dried
1 Step VP41	Lallemand	Temperature: 17°C Alcohol tolerance: high. TSO_2 tolerance: < 60 ppm	High alcohol red wines	Freeze-dried
Enoferm Beta	Lallemand	Temperature: 14°C Alcohol tolerance:15%. pH: > 3.2 TSO_2 tolerance: < 60 ppm	Red wines	Freeze-dried

TABLE 1 (CONTINUED)

A general overview of some of the MLF starter cultures that are available as well as their main characteristics and applications (compiled from company websites).

Lalvin 31	Lallemand	Temperature: 13°C. pH: > 3.1 TSO_2 tolerance: < 45 ppm	Red and white wines	Freeze-dried
Lalvin Elios 1	Lallemand	Temperature: 18°C Alcohol tolerance: high. pH: > 3.4 TSO_2 tolerance: < 50 ppm	Red wine	Freeze-dried
Lalvin ICV Elios Blanc	Lallemand	pH: <3.4	White and rosé wines with difficult pH and temperature conditions	Freeze-dried
Lalvin VP41	Lallemand	Temperature: 16°C Alcohol tolerance: excellent. pH: > 3.1 TSO_2 tolerance: < 60 ppm	High alcohol red wines	Freeze-dried
PN4	Lallemand	Temperature: 16°C pH: > 3. TSO_2 tolerance: < 60 ppm	Red and white wines	Freeze-dried
Lalvin Bacchus	Lallemand	Temperature: 18-24°C Alcohol tolerance: 13.5%. pH: > 3.1	Red and white wines	Freeze-dried
BioStart oenos SK1	Erbslöh Geisenheim	Temperature: 17-25°C Alcohol tolerance: 13%. pH: > 3.1	Simple-structured red and white wines	NA
BioStart Forte SK2	Erbslöh Geisenheim	Temperature: 14-25°C Alcohol tolerance: 14.5%. pH: > 3	Red wine but also suited for white wine	NA
BioStart Bianco SK3	Erbslöh Geisenheim	Temperature: 13-24°C Alcohol tolerance: 13.5%. pH: > 3	White wines with low diacetyl concentration	NA
BioStart Vitale SK11	Erbslöh Geisenheim	Temperature: >16°C Alcohol tolerance: 15.5%. pH: > 3 TSO_2 tolerance: high	Red and white wines	NA

* Total SO_2

** NA: not available

Saccharomyces cerevisiae, ability to survive the production process, the inability to produce biogenic amines, the lack of off-flavour or off-odour production as well as the production of aroma compounds that could potentially contribute to a favourable wine aroma profile (Wibowo *et al.*, 1985; Kunkee, 1991; Fugelsang & Zoecklein, 1993; Henick-Kling, 1993; Le Jeune *et al.*, 1995; Drici-Cachon *et al.*, 1996; Lonvaud-Funel, 2001; Marcobal *et al.*, 2004; Volschenk *et al.*, 2006). Recently, Guzzon *et al.* (2009) selected a new strain for MLF, using the resistance to low fermentation temperature, high SO_2, high ethanol concentration and low pH as selection criteria. The technological and qualitative properties important in the selection criteria for LAB strains for use in starter cultures for MLF were recently summarised by Krieger-Weber (2009).

The procedure of strain selection is a complex and laborious process that involve various screening procedures and trial vinifications. Lactic acid bacteria are isolated from spontaneous fermentations that have natural selective pressures of low pH, low temperature, high alcohol and high SO_2 levels. Individual colonies then undergo vigorous genetic screening to confirm identity, differentiate between strains and determine genetic stability. These strains are then evaluated for their resistance to the physiochemical properties in wine, metabolic properties, nutritional requirements and their ability to survive and retain viability after the drying process. One of the final steps is microvinifications to evaluate the strains under actual winemaking conditions (Bou & Powell, 2006; Mañes-Lázaro *et al.*, 2008a, 2008b, 2009; Capozzi *et al.*, 2010; Ruiz *et al.*, 2010).

Even with the use of commercial starter cultures, complete and successful MLF is not always guaranteed, especially under very difficult wine conditions (i.e. low pH, high ethanol) (Guerzoni *et al.*, 1995). It is imperative that winemakers follow the directions for the reactivation of freeze-dried starter cultures as recommended by the manufacturer, as this minimises some of the potential loss in viability due to direct inoculation in the wine (Davis *et al.*, 1985; Nault *et al.*, 1995; Nielsen *et al.*, 1996; Volschenk *et al.*, 2006). The success of the inoculated bacterial culture to initiate and successfully complete MLF is also influenced by the timing of inoculation. The winemaker should also consider a commercial starter culture that can tolerate the physiochemical properties of

the wine to be inoculated as well as the specifications (e.g. the ability to tolerate high alcohol concentrations) of each culture as reported by the manufacturer.

Timing of inoculation

There are three possible inoculation scenarios for MLF referred to in this review: simultaneous inoculation for AF and MLF (co-inoculation), inoculation during AF and inoculation after the completion of AF (sequential inoculation).

Henick-Kling & Park (1994) and Alexandre *et al.* (2004) mentioned the possible risks of simultaneous inoculation as the development of undesirable/antagonistic interactions between yeast and/or bacteria, stuck AF and the production of possible off-odours. In contrast, Jussier *et al.* (2006) found no negative impact on fermentation success or kinetics associated with simultaneous inoculation, compared to traditional post AF inoculation and no difference in the final wine quality of cool-climate Chardonnay wines. They propose that simultaneous inoculation can be used as a tool to overcome high ethanol levels and reduced nitrogen content at the end of AF. Zapparoli *et al.* (2009) investigated the use of acclimatised bacterial cells in co-inoculation and sequential inoculation as a means to induce MLF in high alcohol wines. Co-inoculation of the bacterial cells resulted in complete MLF in a shorter time period compared to that of the sequential inoculation.

During co-inoculation, the simultaneous metabolism of citric acid and glucose could lead to the production of more acetic acid by *O. oeni*, a heterofermentative LAB (Liu, 2002; Costello, 2006). It has also been shown that wines that have undergone simultaneous AF/MLF tend to be less buttery, retain more fruitiness and are therefore more complex and better structured with marginally higher but sensorial insignificant levels of acetic acid (Henick-Kling, 1993; Bartowsky *et al.*, 2002b; Jussier *et al.*, 2006; Krieger, 2006). Semon *et al.* (2001) and Jussier *et al.* (2006) compared co-inoculation with sequential inoculation in Chardonnay wines. Jussier *et al.* (2006) found no negative impact of simultaneous AF/MLF on the fermentation success or final wine parameters. The sensory panel could not differentiate between wines from the two treatments and, although slightly higher levels of acetic acid were produced in the co-inoculation treatments in both studies, the differences were not statistically relevant and within the range of concentrations normally found in wine. Co-inoculation also had the advantage of reducing overall fermentation duration. Other advantages include more efficient MLF in 'difficult' wines (e.g. low pH) due to low levels of ethanol and higher nutrient concentrations. Wines are also immediately available for racking, fining and SO_2 additions (Davis *et al.*, 1985; Jussier *et al.*, 2006). More recent results on co-inoculation, as mentioned above, highlight this practice as a viable option if care is taken regarding the strain selection of both the bacteria and the yeast.

Inoculation during AF is not a common practice and Rosi *et al.* (2003) reported the strongest antagonism between yeast and bacteria with inoculation of LAB during AF. Bacterial populations showed drastic decreases with this type of inoculation and this could be attributed to various factors including the removal of nutrients by the yeast, accumulation of SO_2, ethanol production, toxic metabolite production by the yeast and acid production by the yeast that decrease the pH. The same study found that at the end of AF, yeast presence favoured the growth and malolactic activity of LAB. This could be attributed to yeast autolysis that release vitamins, amino acids, proteins and polysaccharides that stimulate bacterial metabolism (Henick-Kling, 1993). Early results by some authors advocate sequential inoculation as a means to avoid the problems associated with early inoculation (Ribéreau-Gayon, 1985; Henick-Kling, 1993). The advantages of sequential inoculation include the lack of adverse interactions between yeast and bacteria as well as a reduced risk of acetic acid production due to smaller residual sugar concentrations (Costello, 2006). In spite of these advantages, there are still risks related with sequential inoculation and a loss in viability may possibly be attributed to the presence of high ethanol concentrations, low pH, SO_2, other antimicrobial compounds produced by the yeast as well as nutrient depletion (Larsen *et al.*, 2003).

The timing of inoculation therefore merits careful consideration and will ultimately affect the style and quality of the wine. It is clear that the timing of inoculation for MLF and the concomitant interaction between the yeast and bacterial cultures play an important role in the success of MLF.

FACTORS INFLUENCING MALOLACTIC FERMENTATION

There are various factors that have an effect on LAB and in turn the successful completion of MLF. These factors may directly influence the growth or affect the metabolic properties of LAB. These include pH, temperature, ethanol, SO_2, as well as other products related to yeast metabolism.

Kunkee (1991) listed temperature, ethanol, pH and SO_2 as the four major parameters that would influence the commencement and rate of MLF. This was confirmed by Vaillant *et al.* (1995) that found the same four parameters had the largest inhibitory effect on the malolactic activity of three *O. oeni* strains and three *L. plantarum* strains. Gockowiak & Henschke (2003) suggested that LAB culture viability may be more significantly affected by the wine matrix than wine parameters like pH and ethanol. In addition, it is not only the individual effects of the different factors that have to be taken into account, but the interactive and synergistic effects are also to be considered. These influencing factors do not only affect the growth and the malolactic activity of LAB, but also influence the effect that the LAB will have on wine aroma. Delaquis *et al.* (2000) saw changes in the wine chemistry and aroma characteristics in Chancellor wines and attributed this to the interaction between the LAB culture, yeast strain and fermentation temperature.

From these findings it is clear that there are a selection of factors to consider, including their interactions and the effect of the wine matrix. The following factors will be discussed in more detail: the interaction between yeast (*S. cerevisiae*) and bacteria, yeast-related metabolic products including ethanol and medium chain fatty acids as well as physiochemical wine parameters like pH, temperature and SO_2, the presence of various phenolic compounds, the addition of lysozyme as well as a brief overview of the influence that different vinification procedures have on LAB.

Yeast-bacteria interactions

A factor that the winemaker has the most control over is the selection of the yeast and bacterial culture for AF and MLF, respectively. The interaction between bacteria and yeast during AF and/or MLF will have a direct effect on LAB growth and malolactic activity. Various studies have been done to attempt

an understanding of the interaction between yeast and bacteria (Henick-Kling & Park, 1994; Rosi *et al.*, 2003; Arnink & Henick-Kling, 2005; Guilloux-Benatier *et al.*, 2006; Jussier *et al.*, 2006; Osborne & Edwards, 2006), with a comprehensive review of the possible interactions by Alexandre *et al.* (2004).

Alexandre *et al.* (2004) proposed that the degree and complexity of these interactions are due to three factors. Firstly, the combination of yeast and bacteria strain. In a recent study by Nehme *et al.* (2008) on the interactions between *S. cerevisiae* and *O. oeni* during the winemaking process, it was found that the extent to which inhibition between these microorganisms occur is largely dependent on the selected strains of yeast and bacteria and that the inhibition correlated to a decrease in bacterial growth, rather than a decline in the malolactic activity of the bacteria. In contrast, Arnink & Henick-Kling (2005) in a study of commercial pairings of *O. oeni* and *S. cerevisiae*, found the differences between vintages and grape varieties to be more influential on LAB and MLF than the effect of a particular yeast/bacteria strain combination.

Costello *et al.* (2003) proposed a method for testing the compatibility between yeast and bacteria. The aim of the study was to investigate the interaction between these two microorganism populations without the effect of extrinsic grape-derived or processing factors like SO_2 additions, modified pH, sugar concentration and the presence of pesticide residues or nutrients. A chemically defined medium was used to successfully characterise the metabolic interactions between the yeast and bacteria and replacement of the synthetic media with Chardonnay juice produced similar results. This could be an effective tool for screening yeast/LAB combinations in advance to ensure compatibility and lack of antagonistic or inhibitory effects. The winemaker also has control over the vinification practices applied during the winemaking process. These decisions can also affect the interaction between the bacteria and yeast culture. Table 2 shows the effect that different vinification procedures and decisions have on LAB as well as yeast/bacteria interactions.

The second factor is the uptake and release of nutrients by the yeast, which will in turn affect the nutrients available for the LAB. At the start of AF, *O. oeni* is inhibited by *S. cerevisiae* due to the rapid uptake of certain grape metabolites from the must by the yeast. These compounds include sterols, amino acids and vitamins (Larsen *et al.*, 2003), which result in a nutrient diminished environment for the bacteria. During AF the amino acids and vitamins that are essential for bacterial proliferation are depleted by yeast metabolism to such an extent, that the commencement of bacterial growth is delayed until yeast cells lyse (Nygaard & Prahl, 1997; Alexandre *et al.*, 2004; Arnink & Henick-Kling, 2005). Yeast autolysis plays a vital role in the release of essential nutrients for LAB proliferation and survival (Alexandre *et al.*, 2004). Yeast autolytic activity can release amino acids, peptides, proteins, glucans and mannoproteins and release of these macromolecules are yeast strain dependant (Alexandre *et al.*, 2001, 2004). Mannoproteins seem to be of significant importance, as their release can stimulate bacterial growth by adsorbing medium chain fatty acids and thus detoxifying the wine medium. Mannoproteins can also be enzymatically hydrolysed by bacterial enzyme activity, which will enhance the nutritional content of the wine and in turn stimulate bacterial growth (Guilloux-Benatier & Chassagne, 2003; Alexandre *et al.*, 2004). Yeast metabolism has a direct effect on the nitrogen concentration available for LAB consumption. This was recently confirmed by Guilloux-Benatier *et al.* (2006), who found that proteolytic activity by yeast can affect the nitrogen composition of wine after AF, which in turn will affect the ability of *O. oeni* to grow and complete MLF.

Information on the specific nitrogen compounds that are yeast-derived and that are actually of importance to LAB metabolism, besides amino acids, are limited (Alexandre *et al.*, 2001). It is therefore necessary to identify the essential nutrients for which both LAB and yeast compete and to quantify these compounds to ensure the viability and growth of these microorganisms (Arnink & Henick-Kling, 2005). Metabolic compounds that still warrant further investigation as to their exact role in yeast-bacteria interactions and LAB growth stimulation include vitamins, nucleotides and lipids released by the yeast.

Comitini *et al.* (2005) related part of the inhibitory effect of *S. cerevisiae* to the production of extracellular compounds via metabolic activity of the yeast, rather than a competition for nutrients. Therefore, the third factor to consider is the ability of the yeast to produce metabolites that can either have a stimulatory or inhibitory/toxic effect on LAB. There are a number of yeast-derived inhibitory compounds, including ethanol, SO_2, medium chain fatty acids and proteins. The first three are the compounds most commonly studied with regards to LAB growth inhibition (Alexandre *et al.*, 2004). Osborne & Edwards (2006) found a peptide produced by *S. cerevisiae* inhibited *O. oeni* and that this inhibition is dependent on the presence of SO_2. This study was performed in synthetic medium and the proposed mechanism was the possible disruption of the cell membrane. Similarly, Comitini *et al.* (2005) also reported a LAB inhibitory compound produced by yeast to be heat and protease sensitive and therefore also of a proteinaceous nature. In a similar study, Nehme *et al.* (2010) reported the inhibition of an *O. oeni* strain by *S. cerevisiae* that resulted in a decrease in the malic acid consumption by the LAB strain. This inhibition could be attributed, in part, to a peptidic fraction produced by the yeast. Table 3 provides a summary of the major inhibitory compounds produced by yeast.

To add to the complexity of these interactions, some yeast strains can be both stimulatory and inhibitory, certain LAB strains are capable of inhibiting wine yeast and the composition of the must, as well as vinification practices, influence the interaction.

Ethanol

Ethanol is the main yeast metabolite formed during AF and due to its adverse effect on LAB growth and metabolic activity, plays an integral role in the ability of LAB to survive in the wine environment and accomplish MLF. As with most LAB inhibitory factors, ethanol also demonstrates synergistically inhibiting effects with temperature. The optimal growth temperature of LAB decrease at high ethanol concentrations and elevated temperatures lower the ability of LAB to withstand increased ethanol concentrations (Henick-Kling, 1993; Bauer & Dicks, 2004). Temperatures of 25°C and above, combined with ethanol levels of 10 to 14% (v/v), almost completely inhibit LAB growth and optimum growth at these ethanol levels occur between 18 and 20°C (Henick-Kling, 1993). Capucho & San Ramao (1994) documented no inhibition of the malolactic activity of *O. oeni* with ethanol levels of up to 12% (v/v), but saw an inverse correlation between the growth of

TABLE 2

The influence of different winemaking practices on LAB growth (compiled from Edwards *et al.*, 1990 and Alexandre *et al.*, 2004).

Practice	Influence
Degree of must clarification	Significant impact on bacterial growth. Yeast produce more medium chain fatty acids in highly clarified must
Skin contact prior to AF	Direct effect on extraction of nitrogenous and other macromolecules Stimulate LAB growth and malolactic activity
Choice of yeast strain	Inhibitory and stimulatory effects differ between strains
Ageing of wine on yeast lees	Yeast autolysis release nutrients that stimulate LAB growth and malolactic activity

TABLE 3

Yeast activity inhibiting LAB via the production of yeast metabolites.

Yeast metabolite	Effect on LAB and/or MLF	Reference
Ethanol	Affect growth ability rather than malolactic activity	Alexandre *et al.* (2004)
SO_2	AF with SO_2 producing yeast strain results in wine inhibitory to MLF	Henick-Kling & Park (1994) Alexandre *et al.* (2004)
Medium chain fatty acids	Affect LAB growth and reduce ability to metabolise malic acid. Combination of fatty acids (hexanoic, octanoic and decanoic acid) cause greater inhibition than individual compounds.	Alexandre *et al.* (2004) Edwards *et al.* (1990) Lonvaud-Funel *et al.* (1988)
Metabolites of protein nature	Peptide produced by *S. cerevisiae* during AF: inhibit *O. oeni* by disruption of cell membrane; inhibition dependant on SO_2	Osborne & Edwards (2006) Nehme *et al.* (2010)

O. oeni and increasing ethanol concentrations (Davis *et al.*, 1988; Henick-Kling, 1993; Alexandre *et al.*, 2004; Bauer & Dicks, 2004).

It is generally acknowledged that *O. oeni* strains are able to survive and proliferate in 10% (v/v) ethanol at pH 4.7 (Britz & Tracey, 1990). G-Alegría *et al.* (2004) reported the ability of *O. oeni* and *L. plantarum* strains to grow at 13% (v/v) ethanol and Henick-Kling (1993) stated that ethanol concentrations exceeding 14% (v/v) inhibit the growth of *O. oeni*. The degree to which LAB are able to tolerate ethanol concentrations are strain dependant, as well as being contingent upon the activation steps before inoculation in the wine (Britz & Tracey, 1990).

Chu-Ky *et al.* (2005) investigated the effects of combined cold, acid and ethanol shock on the physical state of the cell membrane and survival of *O. oeni*. Ethanol shocks (10 to 14% v/v) resulted in instantaneous membrane fluidisation followed by rigidification and a decrease in cell viability, whereas the combined ethanol and acid shock of 10% (v/v) and pH 3.5, respectively, resulted in total cell death. In the presence of high concentrations of ethanol the bacteria respond by attempting to maintain the fluidity and integrity of the cell membrane (Couto *et al.,* 1996).

Zapparoli *et al.* (2009) investigated a possible strategy to conduct MLF in wines that generally do not support MLF due to high ethanol concentrations. The study was performed in Amerone wines with an alcohol content of up to 16% (v/v) and both co-inoculation and sequential inoculation were investigated. Complete degradation of L-malic acid was observed with the use of a starter preparation consisting of bacterial cells that were acclimatised in a wine/water mixture for 48 hours prior to inoculation in the wine. Despite the fact that complete MLF occurred under both inoculation scenarios, the sequential inoculated wine took 112 days to complete MLF, compared to 70 days for co-inoculation. Co-inoculation of high alcohol wines with acclimatised bacterial cells could be a valid strategy for conducting complete MLF in potential high alcohol wines, especially in warmer wine regions like South Africa where grapes are harvested with higher sugar concentrations.

The ability of LAB to tolerate elevated concentrations of ethanol is dependant on a number of factors, including temperature and strain selection.

Sulphur dioxide

The addition of SO_2 at crushing and at later stages in the vinification process is an acceptable method for the inhibition and control of microbial populations (Fleet & Heard, 1993). Sulphur dioxide exists in various forms in equilibrium in the wine environment including bound SO_2, molecular or free SO_2 and bisulphite (HSO_3^{-1}) and sulphite (SO_3^{-2}) ions (Fugelsang & Edwards, 1997). The equilibrium of the various SO_2 forms is pH-dependant. At low pH, free SO_2 predominates, consisting mainly of bisulphite and a small fraction of molecular SO_2 and sulphite anions (Usseglio-Tomasset, 1992; Bauer & Dicks, 2004). Molecular SO_2 is considered to be the most inhibitory form, most effective at lower pH values and the only form of SO_2 that can cross bacterial cell walls via diffusion. Inside the cells, the molecular SO_2 is converted to bisulphite and may react with various cell components like proteins and affect the growth of LAB (Carreté *et al.*, 2002; Bauer & Dicks, 2004). Nielsen *et al.* (1996) found that the combination of low pH (pH 3.2) and high SO_2 concentration (26 mg/L) had a strong inhibitory effect on freeze-dried *O. oeni* starter cultures.

The mechanism by which SO_2 inhibit LAB include the rupturing of disulphide bridges in proteins as well as reacting

with cofactors like NAD^+ and FAD, thereby affecting the growth of LAB (Romano & Suzzi, 1993; Carreté *et al.*, 2002). The antimicrobial activity of SO_2 can also influence the malolactic activity (Fornachon, 1963; Wibowo *et al.*, 1985; Henick-Kling, 1993; Lonvaud-Funel, 1999). It has recently been shown that SO_2 is able to inhibit the ATPase activity which is essential in the maintenance of the intracellular pH and therefore LAB growth (Koebmann *et al.*, 2000; Carreté *et al.*, 2002). It has been reported that molecular SO_2 concentrations as low as 0.1-0.15 mg/L may be inhibitory to the growth of some strains. A total SO_2 and bound SO_2 concentration of less than 100 mg/L and 50 mg/L respectively are recommended to ensure successful MLF (Rankine *et al.*, 1970; Powell *et al.*, 2006).

There are various compounds, primarily carbonyl compounds, including acetaldehyde, α-ketoglutaric acid and pyruvic acid, that are able to bind SO_2 resulting in the bound form which demonstrates weaker antimicrobial activity (Henick-Kling, 1993). Besides being sensitive to inhibition by the molecular form of SO_2, LAB also possess the ability to liberate SO_2 from acetaldehyde-bounded sulphur, which then prevents further growth of the bacteria and could result in stuck or sluggish MLF (Fornachon, 1963; Osborne *et al.*, 2006).

LAB species also differ in their ability to tolerate SO_2. Both Davis *et al.* (1988) and Larsen *et al.* (2003) found that *O. oeni* strains were less tolerant to high total SO_2 concentrations than strains of *Pediococcus*.

Besides the addition of SO_2 as part of the vinification process, yeasts are also able to produce significant amounts of SO_2 (King & Beelman, 1986). This ability is dependent on both the media composition as well as the selected yeast strain (Romano & Suzzi, 1993). Most strains produce less than 30 mg/L, although some strains are able to produce, in extreme cases, more than 100 mg/L (Suzzi *et al.*, 1985). Henick-Kling & Park (1994) found that the yeast strains used in their study were able to contribute maximum SO_2 levels of between 13 and 42 mg/L to the total SO_2 concentration, of which the larger amounts had a strong inhibitory effect on LAB growth. In a similar study conducted in Chardonnay, Larsen *et al.* (2003) investigated different wine yeast strains for their ability to inhibit *O. oeni* strains. Yeast strains in this study produced SO_2 concentrations ranging from less than 15 mg/L to 75 mg/L of total SO_2. The yeast also produced very little or no free SO_2. The wines containing higher concentrations of total SO_2 were still generally more inhibitory towards *O. oeni*. Due to the low levels of free SO_2 produced by the yeast, this research suggests that the remaining fraction of bound SO_2 may be more inhibitory than previously considered.

Due to the large influence of wine pH and individual strain tolerance to SO_2, the effect of different SO_2 concentrations is diverse. The type of SO_2 present (free or bound) also influences the effect on LAB, be it a reduction in malolactic activity or a reduction in LAB growth. Henick-Kling (1993) reported a 13% reduction in malolactic activity with 20 mg/L of bound SO_2, a 50% reduction at 50 mg/L and no malolactic activity at 100 mg/L of bound SO_2, while a concentration of 30 mg/L bound SO_2 delayed LAB growth. Lower concentrations of free SO_2 are needed for the inhibition of LAB. In results published by Guzzo *et al.* (1998), *O. oeni* died within 3 hours in 15 mg/L of free SO_2, whereas Carreté *et al.* (2002) found that a free SO_2 concentration of 20 mg/L inhibited LAB ATPase activity by more than 50% and MLF took 40 days to complete in the presence of 5 mg/L free SO_2.

For the control and inhibition of LAB, Henick-Kling (1993) suggests maintaining levels of free SO_2 above 10 mg/L and a total SO_2 concentration of above 30 mg/L. Due to the crucial effect that pH has on the form of SO_2 present, García-Ruiz *et al.* (2008) recommend the following concentrations of free SO_2 to inhibit LAB: 10 to 30 mg/L for pH 3.2 to 3.6, 30 to 50 mg/L for pH 3.5 to 3.7 and 100 mg/L for wines with a pH of over 3.7.

It is essential for the winemaker to not only take the SO_2 added at different stages of the winemaking process into consideration, but also the possible levels of SO_2 produced by the yeast, particularly if MLF is required. The combined SO_2 concentration from these two sources will influence bacterial survival and proliferation as well as MLF initiation (Henick-Kling & Park, 1994; Alexandre *et al.*, 2004). It is important to choose a yeast strain that does not produce significant amounts of SO_2, and if sulphur is required, then only make small additions at crushing. If larger amounts (>30 mg/L) of sulphur is required (e.g. damaged grapes), then MLF inoculation should take place after AF has been completed (Henick-Kling & Park, 1994).

Medium chain fatty acids

Lonvaud-Funel *et al.* (1988) identified medium chain fatty acids (hexanoic, octanoic, decanoic, dodecanoic acid) as one of the main inhibitory products to bacterial growth and MLF formed by yeast metabolism. The inhibitory effects of medium chain fatty acids are highly dependent on the concentration and type of fatty acid (Capucho & San Ramao, 1994; Lonvaud-Funel *et al.*, 1998; Carreté *et al.*, 2002), the choice of both the yeast and bacteria strains (Nygaard & Prahl, 1997) as well as the wine pH, with medium chain fatty acids being more inhibitory at lower pH values (Capucho & San Ramao, 1994; Alexandre *et al.*, 2004).

Medium chain fatty acids have an inhibitory effect on cell growth of LAB and thus the ability of LAB to metabolise malic acid, which in turn leads to an increase in the duration of MLF. The fatty acids inhibit the ATPase activity of LAB and thereby reduce the ability of the bacteria to maintain the intracellular pH and transmembrane proton gradient which is essential for the transport of metabolites across the cell membrane (Capucho & San Ramao, 1994; Carreté *et al.*, 2002).

Lonvaud-Funel *et al.* (1988) found decanoic acid to be inhibitory to both yeast and bacteria and cause yeast-bacteria antagonism, while Carreté *et al.* (2002) reported dodecanoic acid to have the biggest inhibitory effect against *O. oeni*. According to Capucho & San Ramao (1994), decanoic concentrations of above 12.5 mg/L and dodecanoic concentrations of more than 2.5 mg/L inhibited *O. oeni*. Decanoic and dodecanoic acids at concentrations below 12.5 mg/L and 2.5 mg/L, respectively, had a stimulating effect on bacterial growth. In a study by Nehme *et al.* (2008), none of the four yeast strains they studied were able to produce significant levels of medium chain fatty acids. The highest concentrations produced were 24.8 mg/L of octanoic acid, 2.9 mg/L of decanoic acid and 0.2 mg/L dodecanoic acid, which are far below the inhibitory concentrations reported by Capucho & San Ramao (1994).

Selection of the most suitable yeast strain is imperative to the eventual success of MLF in wine. Care should be taken to choose

a yeast strain that is compatible with the strain of LAB, resulting in no or very little antagonistic effects between the yeast/bacteria pairing. This includes a yeast strain that produces very low levels of SO_2 and medium chain fatty acids.

pH

The pH of the wine plays a crucial role in determining the success of MLF. Wines with a pH of 3.3 or higher tend to be less problematic in terms of LAB growth and survival as well as MLF, compared to wines with a lower pH. The LAB species that survive and proliferate in the wine is directly dependant on the pH of the wine (Kunkee, 1967). A pH of 3.5 or lower has a tendency to favour the growth of *O. oeni* and wines with pH levels higher than 3.5, generally favour the growth of *Lactobacillus* and *Pediococcus* species. A pH of less than 3.2 has been shown to be inhibitory to the survival of *O. oeni* (Henick-Kling, 1993). This could be problematic in cooler climate regions where the pH can vary between 2.8 and 3.2 (Liu, 2002).

The wine pH also has a direct effect on the growth rate of bacteria (Kunkee, 1967), with Davis *et al.* (1986a) reporting the inhibition of sugar metabolism and growth of *O. oeni* at low pH. Although the optimum pH for the growth of *O. oeni* is pH 4.3 to 4.8, G-Alegría *et al.* (2004) found that *O. oeni* and *L. plantarum* are able to grow at pH 3.2. Besides influencing bacterial growth, bacterial viability is also affected by wine pH. Gockowiak & Henschke (2003) found a pH of 2.9 to 3.5 to have the largest effect on the bacterial viability of commercial starter cultures of *O. oeni*, similar to Rosi *et al.* (2003) who found that pH 3.2 reduced the bacterial viability of a strain of *O. oeni*. Contrary to these results, Chu-Ky *et al.* (2005) found that, although acid shocks with pH levels of 3 to 4 had an effect on the cell membrane, it did not affect the viability of *O. oeni*. A further effect of pH is the influence on malolactic activity (Henick-Kling, 1993), with the highest malolactic activity seen between pH 3.5 and 4 (Bauer & Dicks, 2004). The pH is also critical to the commencement of MLF as well as the time taken to complete MLF (Rosi *et al.*, 2003). Rosi *et al.* (2003) investigated the effect of pH on *O. oeni* and found the time it took to complete MLF increased with a decrease in pH, with MLF at pH 3.2 and 3.4 taking 15 to 20 days to complete compared to 10 days at pH 3.6.

It is clear that the pH of the wine has a number of decisive affects on MLF and LAB. Besides the direct influence of pH, the relationship between pH and SO_2, as previously discussed, is also crucial in understanding the affect of these parameters on the survival of LAB in wine. Lactic acid bacteria also differ in their ability to tolerate and survive at low pH conditions normally found in wine.

Temperature

Britz & Tracey (1990) investigated the influence of certain factors on the growth of 54 strains of LAB and found that temperature had a profound effect on bacterial growth; ethanol showed the greatest inhibitory effect but there was also a synergistic inhibitory effect in the presence of both ethanol and SO_2.

Temperature is a parameter that is easy to monitor and control, while having a distinct effect on the ability of LAB to survive in wine as well as to initiate and complete MLF. Temperature affects the growth rate, length of the lag phase and population numbers of LAB (Henick-Kling, 1993; Bauer & Dicks, 2004). The optimum growth temperature for *O. oeni* is reported as 27 to 30°C, but due to the presence of alcohol in wine, the optimum growth temperature in wine decreases to between 20 and 23°C (Britz & Tracey, 1990; Henick-Kling, 1993; Bauer & Dicks, 2004; Ribérau-Gayon *et al.*, 2006). The optimum temperature for both *O. oeni* growth as well as malic acid metabolism in wine is 20°C (Ribérau-Gayon *et al.*, 2006). G-Alegría *et al.* (2004) found that both *O. oeni* and *L. plantarum* are able to survive at 18°C, but temperatures below 18°C delay the onset of MLF and increase the duration of MLF, whereas temperatures below 16°C inhibit the growth of *O. oeni* as well as leading to a decrease in cellular activity (Henick-Kling, 1993; Ribérau-Gayon *et al.*, 2006). While lower temperatures (below 16°C) decrease cellular activity, Chu-Ky *et al.* (2005) found that although cold shocks (8 and 14°C) affected the plasma membrane, it did not effect cell survival.

To ensure the rapid initiation and completion of MLF, it is essential to control the fermentation temperature. The fermentation temperature during MLF should be kept at 18 to 22°C to ensure optimum malolactic activity of the LAB.

Nutritional requirements

Besides physiochemical parameters like ethanol, pH, SO_2 and temperature, the nutritional status of the wine is crucial in determining the success of LAB in carrying out MLF and the availability of certain nutrients are therefore imperative (Fugelsang & Edwards, 1997; Théodore *et al.*, 2005). Lactic acid bacteria have been described as 'fastidious' with regards to their nutritional requirements as a result of their limited biosynthetic capabilities (Fugelsang & Edwards, 1997; Théodore *et al.*, 2005; Terrade *et al.*, 2009). One of the main components that play a role in LAB survival is the presence of amino acids and due to the incomplete amino acid biosynthetic ability in LAB, the systems that are responsible for amino acid release via protein hydrolysis, is well developed. It has been shown that LAB are able to release essential amino acids to meet survival- and growth requirements (Matthews *et al.*, 2004). This is an important characteristic seeing as LAB are not able to utilise diammonium phosphate as nitrogen source (Fugelsang & Edwards, 1997). Several essential amino acids have been identified, including glutamic acid, valine, arginine, leucine, isoleucine, as well as cysteine and tyrosine. These may differ according to the bacterial strain (Garvie, 1967; Fugelsang & Edwards, 1997). Earlier studies also identified nicotinic acid, riboflavin, pantothenic acid and either thiamine/ pyridoxine as being essential to bacterial growth. Many species also require purines and folic acid (Du Plessis, 1963; Fugelsang & Edwards, 1997). A recent study by Terrade & Mira de Orduña (2009) investigated the essential nutrient requirements of LAB strains from the *Oenococcus* and *Lactobacillus* genera. It was found that 10 compounds were essential for the growth of all the tested strains and that the essential nutrient requirements are strain specific. These 10 compounds include the carbon and phosphate source, manganese and in accordance with other authors, several amino acids and vitamins. The 'tomato-juice factor' has also been described in literature (Garvie & Mabbitt, 1967). This compound has been described as a derivative of pantothenic acid and although it has not been shown to be essential for all LAB strains, slower bacterial growth has been reported in the absence of this factor (Amachi, 1975; Tracey & Britz, 1987; Fugelsang & Edwards, 1997).

Wines with a low nutrient status will encumber bacterial growth. This situation can be exacerbated by the addition of a yeast strain with a high nutrient demand as well as the fact that certain yeast strains may be prone to producing higher SO_2 concentrations in a nutrient deficient environment (Théodore *et al.*, 2005). It has been proposed that co-inoculation of a malolactic starter culture or the addition of a bacterial nutrient could potentially overcome these difficulties. Strain selection of both the yeast and bacterial culture could be an essential tool to ward of future problems with regards to the nutritional status of the grape must or wine (Jussier *et al.*, 2006).

Phenolic compounds

The major phenolic compounds present in grapes and wine include the non-flavonoids and flavonoids. The non-flavonoids consist of the benzoic- and cinnamic acids and their esters. The flavonoids include the anthocyanins, flavanols, flavan-diols and flavonols (Cheynier *et al.*, 2006).

The amount of phenolics present in wine is cultivar specific as well as being dependant on the vinification procedures implemented by the winemaker (Rozès *et al.*, 2003). The interaction between LAB and phenolic compounds is influenced by various factors including the strain of LAB (Hernández *et al.*, 2007; García-Ruiz *et al.*, 2008) and the type and concentration of phenolic compounds present in the wine (Stead, 1993; Reguant *et al.*, 2000; García-Ruiz *et al.*, 2008). Due to this interaction, phenolic compounds can affect the occurrence as well as rate of MLF (Vivas *et al.*, 1997). Polyphenolic compounds can be transformed by LAB and clear differences in the phenolic content as a result of MLF have been reported (Hernández *et al.*, 2007). The main compounds that can be transformed by different LAB include hydroxycinnamic acids and their derivatives, flavonols and their glycosides, flavanol monomers and oligomers, as well as *trans*-resveratrol and its glucoside (Hernández *et al.*, 2006, 2007).

Hernández *et al.* (2006) investigated the effect of MLF on phenolic compounds in red wine and linked the changes to the metabolism of LAB. The LAB in this study exhibited cinnamoyl esterase activity during MLF with a decrease in the concentration of *trans*-caftaric and *trans*-*ρ*-coutaric acids resulting in a concomitant increase in the corresponding free forms, *trans*-caffeic and *trans*-*ρ*-coumaric acids (hydroxycinnamic acids), respectively. Similarly, Cabrita *et al.* (2008) found that the disappearance of hydroxycinnamoyltartaric acids resulted in an increase in the free forms during both spontaneous and inoculated MLF.

Phenolic compounds can affect bacterial metabolism (Vivas *et al.*, 1997; Rozès *et al.*, 2003), where some phenolic acids inhibit the growth of LAB (Reguant *et al.*, 2000) while others stimulate *O. oeni* (Vivas *et al.*, 1997). García-Ruiz *et al.* (2008) reported the metabolism by LAB of 100 to 250 mg/L of phenolic compounds before inhibition by concentrations exceeding 500 mg/L. Reguant *et al.* (2000) found hydroxycinnamic acids to be inhibitory at high concentrations causing MLF to be delayed by *ρ*-coumaric acid at concentrations of more than 100 mg/L and ferulic acid at concentrations of more than 500 mg/L. Similarly, García-Ruiz *et al.* (2008) reported the use of free hydroxycinnamic acids as a way of controlling *L. plantarum* growth and found ferulic acid to be more inhibitory than *ρ*-coumaric acid, whilst the esters of ferulic acid did not affect growth. Vivas *et al.* (1997) found a slight inhibitory effect on *O. oeni* by vanillic acid, while protocatechuic acid had no effect.

Although the mechanisms by which phenolic compounds inhibit LAB are not entirely clear, there has been some speculation. Possible mechanisms are based on the interactions of phenolic compounds with cellular enzymes (Campos *et al.*, 2003; García-Ruiz *et al.*, 2008) and the adsorption of phenols to cell walls (Campos *et al.*, 2003). Phenolic compounds could lead to a loss in potassium ions, glutamic acid and intracellular RNA, as well as causing a change in the composition of fatty acids (Rozès & Perez, 1998; García-Ruiz *et al.*, 2008). Certain characteristics of wine LAB, like the production of volatile acids and the malolactic activity, are differently affected by the presence of phenolics, and this is dependent on the bacterial strain (Campos *et al.*, 2009).

Phenolic compounds can also have a stimulatory effect on LAB. Free anthocyanins and other phenolic compounds like gallic acid, are able to stimulate cell growth and malic acid degradation of LAB. Phenol carboxylic acids and catechin seem to stimulate the growth of *O. oeni* by enhancing the metabolism of citric acid and reducing the initial lag phase of LAB (Vivas *et al.*, 1997; Rozès *et al.*, 2003). Reguant *et al.* (2000) saw the stimulation of *O. oeni* growth in the presence of catechin and quercitin. Rozès *et al.* (2003) studied the effect of phenolic compounds (the phenolic acids *ρ*-coumaric acid, ferulic, cafeic and gallic acid as well as catechin and the anthocyanin malvidin-3-diglucoside) in a synthetic medium on the growth of *O. oeni*. A concentration of 50 mg/L of phenolic compounds was stimulatory to *O. oeni* growth. This stimulatory effect could be attributed to the role that phenolic compounds play in protecting bacterial cells from ethanol as well as the fact that phenolic compounds reduce the redox potential of the wine which promotes cell growth (Rozès *et al.*, 2003).

The presence of phenolic compounds also has the potential to influence certain quality parameters in wine. Cavin *et al.* (1993) reported the ability of LAB to metabolise hydroxycinnamic acids which result in the formation of volatile phenols with the potential to produce off-flavours. A strain of *O. oeni* studied by Campos *et al.* (2009), was able to produce higher concentrations of acetate in the presence of phenolic acids. This could be due to enhanced citric acid metabolism at the expense of sugar consumption as documented by Rozès *et al.* (2003). It was also found that this phenomenon is strain dependant. In contrast, Reguant *et al.* (2000) found that gallic acid was able to delay or totally inhibit the formation of acetic acid from citric acid. Tannase activity has also been found in *L. plantarum* strains (not in *O. oeni*). Tannase activity allows the hydrolysis of ester bonds in hydrolysable tannins. This reaction releases gallic acid and glucose. Tannase activity could potentially play a role in reducing astringency and haze formation in wine (Vaquero *et al.*, 2004).

The effect that phenolic compounds have on LAB metabolic activity and growth, seem to be dependent on the type of compound and its concentration, as well as the strain of LAB.

Lysozyme

Lysozyme is an enzyme obtained from hen egg white which has been proposed as an alternative to SO_2 for the control of LAB and to delay MLF. This enzyme is highly effective against Gram-positive bacteria (McKenzie & White, 1991; Gerbaux *et al.*,

1997; Bartowsky, 2003; Bartowsky *et al.*, 2004). The enzyme acts by splitting the β-(1-4) linkage between N-acetyl muramic and N-acetyl-glucosamine, components of the peptidoglycan in the bacterial cell wall, leading to cell lysis and death (McKenzie & White, 1991; Bartowsky *et al.*, 2004). Both the susceptibility of LAB, as well as the dosage of lysozyme, is important in determining the efficiency of lysozyme in inhibiting LAB and MLF (Bartowsky, 2003).

Not many studies have been done regarding lysozyme and wine. In a model wine, Green & Daeschel (1994) found ethanol to repress lysozyme activity as well as noting the formation of complexes between lysozyme and phenolics, similar to the lysozyme-phenolic precipitate observed by Bartowsky *et al.* (2004). Gerbaux *et al.* (1997) evaluated the ability of lysozyme to reduce the LAB population in wine after the completion of MLF. An addition of 500 mg/L lysozyme inhibited MLF and an addition of 250 mg/L promoted microbial stability in red wines after MLF was complete. An added observation of oenological importance was the lack of an increase in acetic acid concentrations in wines that were treated with lysozyme, which were confirmed in results obtained by Gao *et al.* (2002). Goa *et al.* (2002) investigated the use of lysozyme in inhibiting spoilage LAB (*Lactobacillus kunkeei*, *L. brevis*, *P. parvulus* and *P. damnosus*) in Chardonnay. Besides having no inhibitory effect on yeast growth or sugar metabolism, lysozyme was extremely effective in inhibiting the growth of all the LAB cultures.

In a study by Bartowsky *et al.* (2004), lysozyme stability as well as the sensorial impact of lysozyme in bottled wines of Riesling, Cabernet Sauvignon and Shiraz were investigated. A fine red coloured pigment was observed in the Cabernet and Shiraz, likely due to the formation of complexes between lysozyme and coloured phenolic pigments. While no precipitate was observed in the Riesling, the lysozyme did cause heat instability or haze formation in the white wine. There were no detectable lysozyme activity in the red wines after two days, attributed to the complex formation and precipitation, while up to 82% residual activity remained in the Riesling after six months. Despite the fact that a colour difference could be observed due to the pigment precipitation, the sensory panel could not distinguish between the lysozyme treated and untreated wines based on wine aroma and flavour.

The use of lysozyme is an alternative option to SO_2 for the control and inhibition of the indigenous LAB population. Lysozyme, in countries where the addition has been legalised, can be utilised as an added tool during the winemaking process to ensure microbial stability with regards to the presence of LAB. An added benefit is the lack of sensory changes associated with the use of lysozyme.

IMPACT OF MALOLACTIC FERMENTATION ON WINE AROMA

Various studies have shown that MLF has the potential to alter the aroma profile of a wine by the modification or production of flavour-active compounds as depicted in Fig. 1 (Davis *et al.*, 1985; Lonvaud-Funel, 1999; Maicas *et al.*, 1999; Nielsen & Richelieu, 1999; Gámbaro *et al.*, 2001; Bartowsky *et al.*, 2002b; Bartowsky & Henschke, 2004; D'Incecco *et al.*, 2004; Swiegers *et al.*, 2005).

Jeromel *et al.* (2008) found MLF wines to be preferred compared to non-MLF wines and as being more round and full in taste. This is in agreement with findings by Herjavec *et al.* (2001), that wines in which MLF was suppressed, were inferior compared to wines that were subjected to inoculated or spontaneous MLF. In contrast to these studies, Sauvageot & Vivier (1997) found that MLF had a minimal, though significant, effect on the aroma of Pinot noir and Chardonnay wines. A possible reason could be that the sensory evaluation of the wines in this study took place two to three years after bottling. This extensive bottling ageing period could have resulted in the modification of the wine aroma profile.

Bartowsky *et al.* (2002b) compiled a list of descriptors used in the sensory analysis of wines that had undergone MLF. Compared to the control wine that had not undergone MLF, all the wines were readily distinguishable based on these descriptors, which included buttery, nutty, vanilla, fruity, vegetative, toasty and wet leather amongst others. The general consensus was that MLF resulted in a creamier palate, less fruit intensity and more butteriness. In contrast, Henick-Kling (1993) found that MLF enhanced the fruity notes, as well as the buttery aroma, and reduced the vegetative, green and grassy aromas, possibly due to the catabolism of aldehydes (Liu, 2002).

Bartowsky & Henschke (1995) proposed three mechanisms by which LAB are able to modify wine aroma and flavour: firstly, the bacteria are able to produce volatile compounds by metabolising grape constituents e.g. sugars and nitrogen containing compounds like amino acids; secondly, the modification of grape or yeast derived secondary metabolites by the bacteria and thirdly, adsorption to the cell wall or metabolism of flavour compounds.

There are various important factors to consider when investigating the effect that MLF and LAB have on wine aroma. The changes in aroma and flavour profiles during MLF are also dependant on the bacteria strain responsible for MLF (Bartowsky & Henschke, 1995; Costello, 2006), as well as on the grape cultivar and winemaking practices (Bartowsky *et al.*, 2002b). One of the most important factors is the matrix effect, where the perception of wine aroma compounds will be significantly altered and effected by the chemical surroundings (Bartowsky *et al.*, 2002b). This implies that an odour-impact compound is not necessarily defined by the concentration at which it occurs in the wine, but rather its threshold value and the contribution that the specific compound makes to the aroma perception of the wine. Other important factors include bacteria-yeast interactions, which also link to the timing of inoculation, precursor availability and enzymatic activity of the malolactic bacteria, as well as whether MLF is completed in a barrel and/or tank.

This section will focus on the main aroma compounds associated with MLF that contribute to the general aroma profile of the wine, as well as some of the key factors that influence their formation. The groups of compounds that will be discussed include carbonyl compounds, esters, sulphur- and nitrogen containing compounds, volatile phenols and volatile fatty acids. A number of these compounds are considered more important due to their larger contribution to the sensory profile and will be discussed in more detail.

Carbonyl compounds

Diacetyl (2,3-butanedione) is a diketone that contributes buttery, nutty and butterscotch characters to the wine, as well as a yeasty character to sparkling wines, during MLF (Bartowsky &

Henschke, 1995; Martineau *et al.*, 1995; Bartowsky *et al.*, 2002b; Bartowsky & Henschke, 2004). It is considered one of the most important aroma compounds produced during MLF (Bartowsky & Henschke, 1995; Lonvaud-Funel, 1999). Diacetyl is formed as an intermediate during the metabolism of citric acid by the LAB present during MLF (Fig. 2) (Bartowsky *et al.*, 2002b; Bartowsky & Henschke, 2004). During carbohydrate metabolism by LAB, pyruvate is reduced to lactate to maintain the redox balance of the bacterial cell. When additional pyruvate is produced as a result of the citric acid metabolism in the absence of sugar, pyruvate is redirected to the production of acetoin and butanediol. Pyruvic acid is reductively decarboxylated to diacetyl via α-acetolactate (Lonvaud-Funel, 1999; Bartowsky *et al.*, 2002b; Bartowsky & Henschke, 2004; Costello, 2006). Due to the fact that diacetyl is chemically unstable, it is further reduced to acetoin, which in turn can be reduced to 2,3-butanediol (Bartowsky *et al.*, 2002b; Costello, 2006).

The accumulation of diacetyl and acetoin is dependent on the rate of MLF. Lower levels of diacetyl and acetoin are produced by a higher MLF rate. Maicas *et al.* (1999) found decreased levels of diacetyl after MLF, but increased levels of 2,3-butanediol due to the enzymatic reduction of diacetyl by LAB. This conversion has a direct effect on wine aroma, due to the fact that acetoin and 2,3-butanediol have higher threshold values, approximately 150 mg/L (Francis & Newton, 2005) and 600 mg/L (Bartowsky & Henschke, 2004), respectively, and are therefore considered to contribute to the buttery aroma to a lesser extent (Bartowsky *et al.*, 2002b). In contrast, diacetyl has an odour threshold of approximately 0.2 mg/L in Chardonnay, 0.9 mg/L in Pinot noir and 2.8 mg/L in Cabernet Sauvignon (Martineau *et al.*, 1995). Francis & Newton (2005) reported diacetyl levels of 0.2 to 1.84 mg/L generally found in young red wines and 1.25 to 3.39 mg/L in aged red wines. When concentrations exceed 5-7 mg/L, the buttery attribute is overpowering and this character is seen as undesirable, whereas concentrations between 1-4 mg/L can contribute to the buttery and butterscotch aroma and add to the complexity of the wine (Bartowsky & Henschke, 1995, 2004; Swiegers *et al.*, 2005). The sensory perception of diacetyl is highly dependent on a number of factors, including the style, age and type of wine (Swiegers *et al.*, 2005; Costello, 2006), as well as the presence of other compounds that are able to react with diacetyl e.g. SO_2 (Martineau *et al.*, 1995; Bartowsky *et al.*, 2002a; Bartowsky & Henschke, 2004; Swiegers *et al.*, 2005). Table 4 lists the various ways to manipulate the diacetyl content during the winemaking process.

The factors that influence the diacetyl concentration provide a tool for manipulating the final diacetyl concentration in the wine, as well as the impact it has on the final wine aroma (Bartowsky *et al.*, 2002b). Citric acid metabolism only commences towards the end of MLF during sequential AF/MLF when most of the malic acid has been converted to lactic acid. This implies that the maximum concentration of diacetyl will occur at the point where the malic acid is depleted (Bartowsky & Henschke, 1995, 2004; Nielsen & Richelieu, 1999). Nielsen & Richelieu (1999) reported on the relationship between diacetyl and SO_2 concentrations in wine during and after MLF. The reaction between SO_2 and diacetyl is exothermic and reversible. With the initial addition of SO_2 upon completion of MLF, the SO_2 binds to diacetyl with a concomitant decrease in the diacetyl concentration. During storage, the reaction is reversed with the resulting increase in diacetyl levels.

By choosing a bacteria strain that possess the ability to produce higher levels of diacetyl, in conjunction with manipulating the temperature, SO_2 content and lees contact during the vinification process, a winemaker can manipulate the diacetyl content according to the style of wine required. Some of these factors have a symbiotic effect. A lower pH will result in more SO_2 present in the active antimicrobial form, which will inhibit yeast and bacteria activity and stabilise the diacetyl content. Air contact during MLF will result in a higher wine redox potential which will facilitate the formation of diacetyl from its precursor. The reaction catalysed by pyruvate decarboxylase, responsible for the decarboxylation of pyruvic acid, requires oxygen. Air exposure during MLF will therefore directly influence the metabolic pathway.

Esters

Esters are important in determining wine aroma and are associated with fruity aromas in wine. The two main groups of fermentation-derived esters that have been associated with wine fruitiness are acetate esters and ethyl fatty acid esters. Ethyl fatty acid esters are formed by the enzymatic esterification of activated fatty acids formed during lipid biosynthesis. Acetate esters are formed through the condensation of higher alcohols with acetyl-CoA (Matthews *et al.*, 2004; Ugliano & Henschke, 2008). Even though the esterase activity of LAB are still being evaluated, it is clear that MLF and wine LAB have the ability to alter the ester content (Matthews *et al.*, 2004). The extent of this alteration is still unclear, with both increases and decreases in ester concentrations being observed in the literature. Malolactic fermentation is generally associated with increased concentrations of ethyl esters, including ethyl lactate, ethyl acetate, ethyl hexanoate and ethyl octanoate (De Revel *et al.*, 1999; Delaquis *et al.*, 2000; Liu, 2002; Swiegers *et al.*, 2005; Jeromel *et al.*, 2008), as well as diethyl succinate. The modulation of aromatic esters by microbial populations has recently been reviewed by Sumby *et al.* (2009). Table 5 contains some of the esters, other than ethyl lactate and diethyl succinate, associated with MLF and possible aromas that they can contribute to wine.

The most important esters that typically play a role in MLF, are ethyl lactate and diethyl succinate (Maicas *et al.*, 1999; Herjavec *et al.*, 2001; Ugliano & Moio, 2005). Ethyl lactate is the esterification product of lactate produced by LAB during MLF and ethanol present as a result of AF. This compound is beneficial to the aroma profile due to its fruity, buttery and creamy aromas as well as its contribution to the mouthfeel of the wine (Ugliano & Moio, 2005). Lloret *et al.* (2002) determined the aroma threshold of (*S*)-ethyl lactate in wine as 110 mg/L. Wines that had not been subjected to MLF had levels of 5 to 8 mg/L, compared to 90 to 150 mg/L in MLF wines. Succinic acid is formed as a by-product of microbial α-ketoglutarate metabolism, which in turn is slowly and non-enzymatically esterified to form diethyl succinate (Ugliano & Moio, 2005). This ester also contributes fruity and melon aromas to the wine and has an odour threshold of 1.2 mg/L (Peinado *et al.*, 2004). Herjavec *et al.* (2001) found a significant increase in diethyl succinate and ethyl lactate after MLF in Riesling wines, accompanied by a decrease in isoamyl acetate, isobutyl acetate, ethyl butyrate and ethyl caproate. Similarly, Ugliano & Moio

TABLE 4

Factors which influence the diacetyl content of wine (Martineau *et al.*, 1995; Bartowsky *et al.*, 2002b; Bartowsky & Henschke, 2004; Saguir *et al.*, 2009).

Influencing Factors	**Effect on diacetyl concentration and/or sensory perception**
LAB strain	LAB strains vary in their diacetyl production potential
Wine type	Red wine favours diacetyl production compared to white wine
Inoculation rate of MLF bacteria	Lower inoculation rate (10^4-10^5 cfu/mL) favours diacetyl production
Contact with actual yeast culture and lees	Yeast contact reduces diacetyl content of wine
Contact of wine with air during MLF	Oxygen favours oxidation of α-acetolactate to diacetyl
SO_2 content	SO_2 binds diacetyl which renders it sensory inactive SO_2 addition inhibits yeast/LAB activity and stabilises diacetyl content at time of addition
Citric acid concentration	Favours diacetyl production, however acetic acid is also produced
Temperature at which MLF is conducted	18°C vs. 25°C may favour diacetyl production
Wine pH at which MLF is conducted	Lower pH may favour diacetyl production
Fermentable sugar concentration	Conflicting information; residual sugar may reduce diacetyl production
Wine stabilisation	Immediate stabilisation after malic and citric acid metabolism will increase diacetyl content

TABLE 5

Concentrations, aroma descriptors and thresholds of some of the other esters found in wine that contribute to the aroma during MLF (compiled from Peinado *et al.*, 2004; Francis & Newton, 2005; Vilanova & Martínez, 2007).

Ester	**Aroma descriptor**	**Concentration (µg/L) in**		**Odour Threshold (µg/L)**
		Young red wine	**Aged red wine**	
Ethyl hexanoate	Apple, fruit, banana, brandy	153 - 622	255 - 2556	5 - 14
Ethyl octanoate	Fruit, sweet, floral, banana, pear	138 - 783	162 - 519	2 - 5
Ethyl butyrate	Apple, fruit, pear, banana	69.2 - 371	20 - 1118	20
Isoamyl acetate	Banana, fruity, sweet	118 - 4300	249 - 3300	30
Phenylethyl acetate	Rose, honey, tobacco, flowery	0.54 - 800	–	250

– not reported above threshold in any study.

(2005) found significant increases in ethyl lactate and diethyl succinate.

Maicas *et al.* (1999) found increases and decreases in the ester concentration as a result of bacterial strain selection. They reported increases in isoamyl acetate, ethyl caproate and 2-phenylethyl acetate. Similarly, Gámbaro *et al.* (2001) found that ethyl- and acetate ester levels decreased during MLF, but these changes were also dependant on the strain of bacteria used. This coincided with a significant decrease in sensory descriptors like 'berry fruit' and 'fresh vegetative'. Jeromel *et al.* (2008) also saw a decrease in isoamyl acetate and 2-phenylethyl acetate due to MLF. Delaquis *et al.* (2000) found an increase in the concentration of ethyl acetate and 3-methyl-1-butyl, which was influenced by the choice of LAB culture. In contrast, ethyl 2-hydroxypropanoate was not influenced by the choice of LAB culture. Ugliano & Moio (2005) studied the effect of four different malolactic starter cultures of *O. oeni* on the concentration of yeast-derived volatile compounds. Malolactic fermentation increased levels of C_4-C_8 ethyl fatty acid esters and 3-methylbutyl acetate, depending on the bacteria strain used. The total increase in ethyl fatty acid esters were generally larger than the increase observed for acetate esters.

Generally, a bacteria strain that exhibits esterase activity contributes to the overall fruitiness of wine and thus the changes in aroma associated with the production and hydrolysis of esters, are dependent on the selected bacteria strain. The majority of *O. oeni* and *Lactobacillus* strains evaluated by Davis *et al.* (1988) showed esterase activity and similarly, all of the strains screened by Matthews *et al.* (2006) could hydrolyse esters. The most activity was noticed in *O. oeni* strains, followed by *Lactobacillus* and *Pediococcus* strains, respectively. Matthews *et al.* (2007) found that esterase showed greater activity towards short-chained esters (C_2 to C_8) in comparison to long chained esters (C_{10} to C_{18}) and significant activity levels still remained under wine-like conditions. These results highlight the fact that esterases originating from LAB could contribute to the wine aroma, if the enzymes are produced and active under wine conditions.

Glycosides

Many volatile aroma compounds are present in the grape bound to a sugar moiety (D'Incecco *et al.*, 2004). These compounds are non-volatile in this glycosidic form and represent a reservoir of potential aroma compounds that could make a contribution to the overall perception of wine aroma, if they are released (Bartowsky *et al.*, 2004; D'Incecco *et al.*, 2004; Swiegers *et al.*, 2005). These potential volatiles and sensory important compounds include monoterpenes, C_{13}-norisoprenoids, benzene derivatives and aliphatic compounds (Sefton *et al.*, 1993; D'Incecco *et al.*, 2004; Matthews *et al.*, 2004). Lactic acid bacteria, primarily *O. oeni*, demonstrate glycosidic activity with the ability to release these volatile compounds so they become odour-active (Grimaldi *et al.*, 2000; Boido *et al.*, 2002; Liu, 2002; Barbagallo *et al.*, 2004; D'Incecco *et al.*, 2004; Matthews *et al.*, 2004). Recently, it has been demonstrated that *Lactobacillus* and *Pediococcus* species also possess glycosidase activity (Grimaldi *et al.*, 2005a; Spano *et al.*, 2005). *Oenococcus oeni* and *Lactobacillus* strains studied by Hernandez-Orte *et al.* (2009) were able to release terpenes, norisoprenoids, phenols and vanillins from glycosidic precursors in a model wine solution. The small increments in the concentrations of these compounds caused a broad change in the aroma profile of the samples.

McMahon *et al.* (1999) found no glycosidase activity in commercial *O. oeni* cultures, whereas Mansfield *et al.* (2002) saw β-glucosidase activity in a model system, but none of the strains were active on Viognier grape glycosides. This could imply that the cultivar has an influence on the enzyme activity. In contrast, Grimaldi *et al.* (2000), Ugliano *et al.* (2003) as well as Ugliano & Moio (2006) found a decrease in the concentration of total glycosides and an increase in the free compounds after MLF with *O. oeni*. Boido *et al.* (2000) found that due to the β-glucosidase activity of *O. oeni*, the free aroma compounds released from their glycosylated forms increased. They postulated that the increase was smaller than expected due to stable associations between released aroma compounds and bacterial polysaccharides. This could be a possible cause as to why D'Incecco *et al.* (2004) observed limited liberation of aroma compounds in Chardonnay glycosidic extract during MLF. The degree to which the enzymatic hydrolysis takes place is dependent on the bacterial strain, chemical structure of the substrate and growth phase of the bacteria. Glycosidase activity is also influenced by pH, temperature, sugars and ethanol (Grimaldi *et al.*, 2000, 2005b). The acidic conditions found in wine may denature or inhibit the enzymatic activity. However, *O. oeni* retained up to 80% of β-glucosidase activity at pH 3.5 (Grimaldi *et al.*, 2000). Barbagallo *et al.* (2004) also showed the ability of wild *O. oeni* strains to retain their β-glucosidase activity under wine conditions. Mtshali *et al.* (2010) screened and characterised the β-glucosidase enzyme in LAB isolated from South African wines. The enzyme specific primers amplified the gene with a size corresponding to 1392 bp, with 40% of the isolates testing positive for the presence of the gene, none of which were *O. oeni* strains. This supports the investigation of alternative LAB genera for possible use in a starter culture that could assist in the liberation of grape-derived aroma compounds.

It is important to further investigate the effect that various stress factors like ethanol and SO_2 could have on enzymatic activity and to choose starter cultures that can make a positive contribution to MLF aroma.

Volatile sulphur compounds

Sulphur containing compounds associated with MLF as a result of LAB metabolism, have not been investigated until as recently as 2004. Pripis-Nicolau *et al.* (2004) were the first to demonstrate the ability of wine LAB to metabolise methionine to produce volatile sulphur compounds during MLF. The formation of volatile sulphur compounds in fermented foods has recently been reviewed by Landaud *et al.* (2008), including volatile sulphur compounds associated with wine. The precise mechanism and biochemical pathways that make up the sulphur metabolism in wine LAB have not been fully investigated and little is known. Figure 3 displays the sulphur metabolism in LAB and it is assumed that wine LAB will share some of the characteristics and pathways characterised in other LAB, specifically LAB from the dairy industry (Liu *et al.*, 2008).

Vallet *et al.* (2008) proposed the possible pathway by which these compounds are formed by *O. oeni*. The metabolism of methionine by LAB leads to the formation of methanethiol, dimethyl disulphide, 3-(methylsulphanyl)propan-1-ol (also known as methionol) and 3-(methylsulphanyl)propionic acid. The formation of these compounds plays an important role in the complexity of wine aroma because of their characteristic and powerful odours shown in Table 6. Increased concentrations of these sulphur compounds will impart negative aromas to the wine, but concentrations below or close to the odour threshold will add to complexity. The threshold values of some of the most important sulphur compounds are listed in Table 6. Concentrations of methanethiol and 3-(methylsulphanyl)propan-1-ol above their thresholds are usually associated with reduction

TABLE 6

The four main volatile sulphur compounds produced by LAB during MLF and their possible contribution to the wine aroma (Landaud *et al.*, 2008).

Compound	Aroma descriptor	Odour threshold in wine (ppb)	Probable precursor	Concentration in wine (ppb)
Methanethiol	Cooked cabbage, onion	0.3	Methionine	2.1-5.1
Dimethyl disulfide	Cooked cabbage, ntense onion	15-29	Methanethiol	2
3-(methylsulphanyl)propan-1-ol	Cauliflower, cabbage	500	Methionine	140-5000
3-(methylsulphanyl)propionic acid	Chocolate, roasted	244	Methionine	0-1811

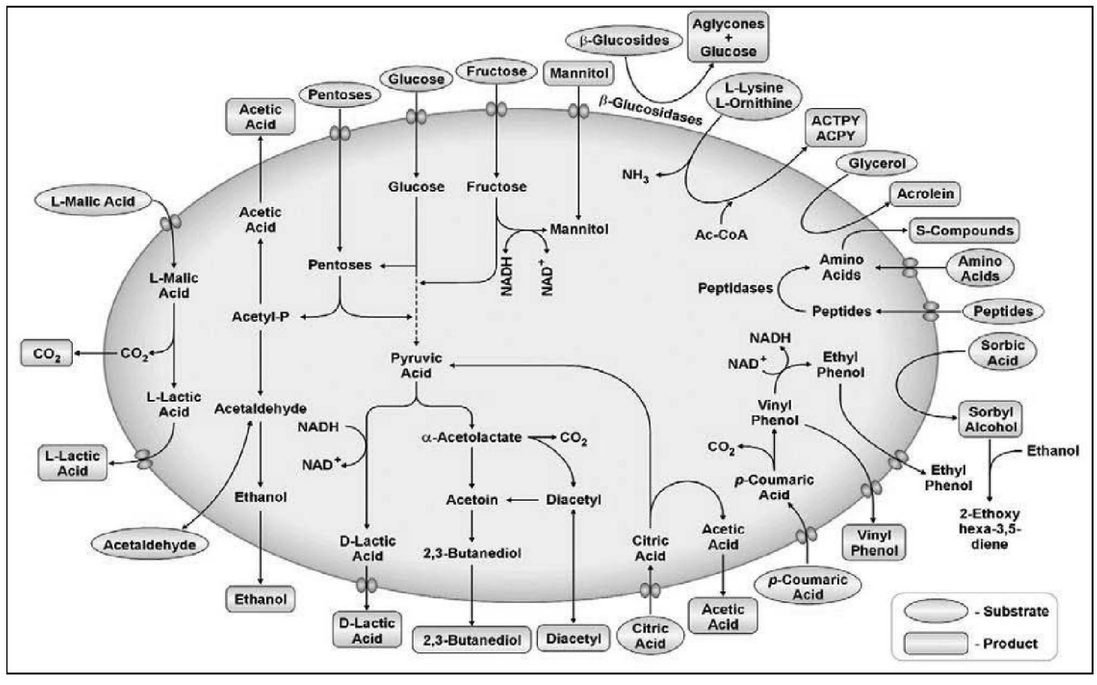

FIGURE 1

A schematic representation of the production and modification of flavour-active compounds by LAB (Swiegers *et al.*, 2005).

off-flavours (Pripis-Nicolau *et al.*, 2004). The production of these volatile sulphur compounds are strain dependant as well as genus dependant, with *O. oeni* having a higher capacity for producing these compounds, compared to the species of *Lactobacillus* (Pripis-Nicolau *et al.*, 2004).

There are various factors that influence the production of these volatile sulphur compounds, including the presence of methionine as precursor and the growth phase of the bacteria. Vallet *et al.* (2008) found the production of methionol occurred during the exponential growth phase of the LAB, while the production of 3-(methylthio)propionic acid took place during both the exponential and stationary growth phase. These authors also found that methionol and 3-(methylthio)propionic acid production only occur in the presence of methional, which implies that this compound is an important precursor in their production.

Methional and 2-oxo-4-(methylthiol) butyric acid (KMBA) are the intermediate compounds that are integral in the production of volatile sulphur compounds by *O. oeni*. Methional is converted to methionol and 3-(methylthiol)propionic acid, and can also be produced from the oxidative decarboxylation of KMBA. KMBA also serves as a precursor for the formation of methanethiol and dimethyl disulphide (Vallet *et al.*, 2008). Pripis-Nicolau *et al.* (2004) found that LAB were able to form all four products in laboratory media, but only 3-(methylsulphanyl)propionic acid levels increased significantly in Merlot red wines. The influence and presence of other compounds in wine have a significant effect on the perceived aroma of 3-(methylsulphanyl)propionic acid. In a synthetic solution, the perception threshold of 3-(methylsulphanyl) propionic acid is 50 µg/L and denotes chocolate and roasted aromas. In contrast, the perception threshold in wine is almost five times higher, 244 µg/L, and is associated with 'earthy' and 'red fruit' sensory descriptors.

Besides the four volatile sulphur compounds discussed here, there are also other sulphur containing compounds which potentially alter wine aroma. It is possible for reactions to take place between the sulphur-containing cysteine and α-dicarbonyl compounds like diacetyl. These are non-enzymatic reactions that usually take place after MLF and produce tetramethylpyrazine and trimethyloxazole that is associated with 'toasted', 'sulphur' and 'cabbage' aromas (Pripis-Nicolau *et al.*, 2000; Swiegers *et al.*, 2005; Landaud *et al.*, 2008).

Nitrogen containing compounds

Wine LAB are able to produce heterocyclic volatile nitrogen bases responsible for the 'mousy' off-flavours encountered in spoilage wines. Three main compounds are involved: 2-acetyl-1-pyrroline (ACPY), 2-ethyltetrahydropyridine (ETPY) and 2-acetyltetrahydropyridine (ACTPY) (Lonvaud-Funel, 1999; Costello *et al.*, 2001). The formation of these compounds is mostly associated with heterofermentative LAB, such as *O. oeni*, some *Lactobacillus* species and *Leuc. mesenteroides*, via the metabolism of certain amino acids, specifically ornithine and lysine (Costello *et al.*, 2001; Swiegers *et al.*, 2005). ACTPY and ACPY are the most powerful odorants compared to ETPY, with thresholds in water of 1.6 µg/L and 0.1 µg/L, respectively. Wines that are considered spoiled by the 'mousy' off-flavours generally contain amounts of 2.7 to 18.7 µg/L of ATPY, up to 7.8 µg/L ACPY and 4.8 to 106 µg/L of ACTPY. These compounds are either present in combination or individually. The availability of the precursor's lysine and ornithine has a significant impact on the ability of LAB to produce these compounds (Costello & Henschke, 2002). Costello & Henschke (2002) also found that the lack of ethanol drastically reduces the ability of *L. hilgardii* to produce N-heterocycles, whereas elevated concentrations of acetaldehyde stimulates formation. Lactic acid bacteria differ in their preference

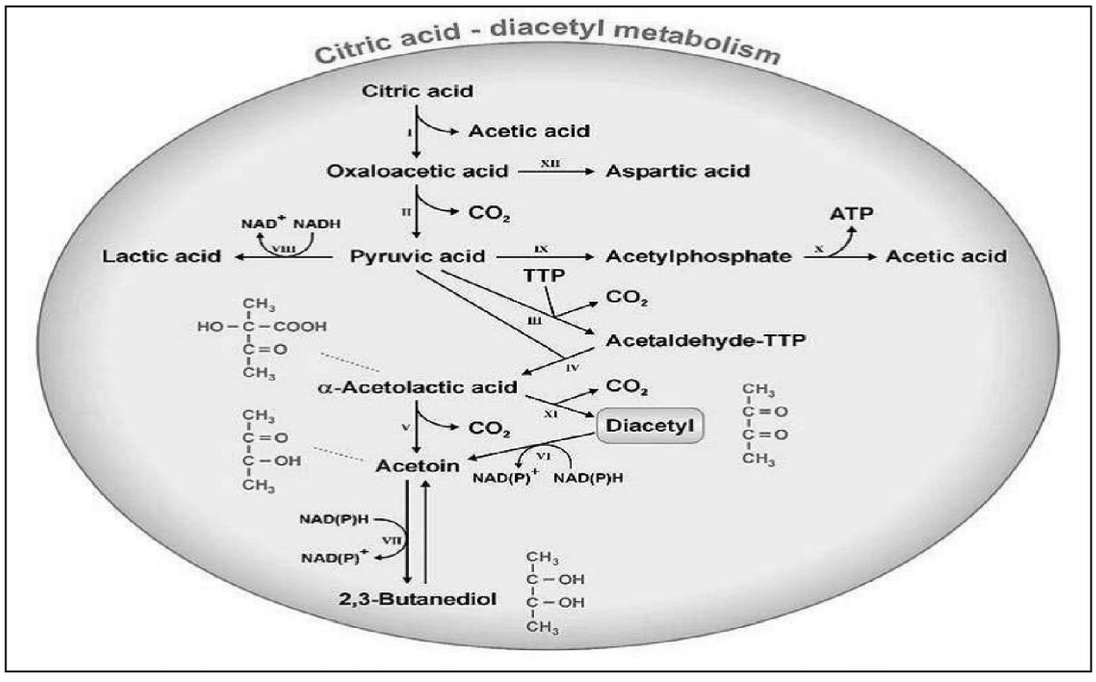

FIGURE 2

A schematic representation of citric acid metabolism and the synthesis of diacetyl in LAB (I: citrate lyase; II: oxaloacetate decarboxylase; III: pyruvate decarboxylase; IV: α-acetolactate synthase; V: α-acetolactate decarboxylase; VI: diacetyl reductase; VII: acetoin reductase; VIII: lactate dehydrogenase; IX: pyruvate dehydrogenase complex; X: acetate kinase; XI: non-enzymatic decarboxylation; XII: aspartate aminotransferase) (Swiegers *et al.*, 2005).

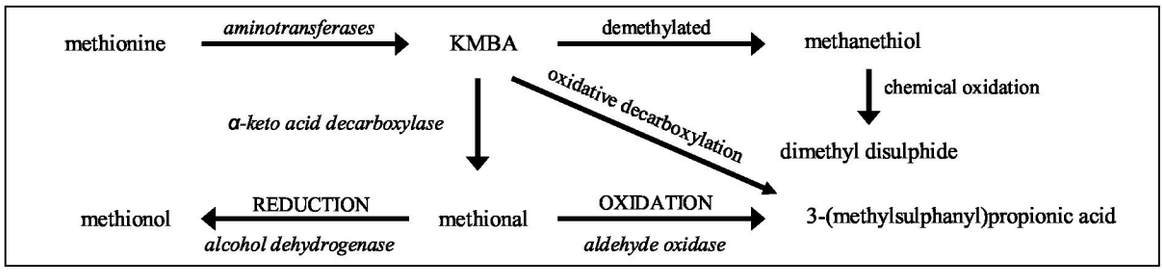

FIGURE 3

The production of the four major volatile sulphur compounds produced by LAB metabolism [Compiled from Landaud *et al.* (2008) and Vallet *et al.* (2008)].

for the formation of the different nitrogen heterocyclic compounds. *Oenococcus oeni* favours the production of the least flavour active ETPY, the heterofermentative lactobacilli the formation of ACTPY and the homofermentative pediococci the formation of the most flavour active ACPY. In general, the heterofermentative LAB show the highest ability to produce nitrogen-heterocycles and mousy off-flavours (Swiegers *et al.*, 2005).

Volatile phenols

Wine contain various phenolic compounds, of which the phenolic acids, specifically *p*-coumaric acid and ferulic acid, can be utilised as substrates by wine LAB in the formation of volatile phenol aroma compounds (Cavin *et al.*, 1993; Lonvaud-Funel, 1999). The bacteria are able to make use of an active transport mechanism to transfer the phenolic acids into the cell, where hydroxycinnamic acid decarboxylases are able to decarboxylate the phenolic acids to their vinyl derivatives (4-vinylguaiacol and 4-vinylphenol). In turn, the vinyl derivatives can be enzymatically reduced to the corresponding ethyl derivatives (4-ethylguaiacol and 4-ethylphenol) (Cavin *et al.*, 1993; Swiegers *et al.*, 2005). The vinyl derivatives can impart pharmaceutical odours to the wine (Ribéreau-Gayon *et al.* 2002) and the products, 4-ethylphenol and 4-ethylguaiacol, give rise to 'animal' and 'medicinal' aromas as well as horse sweat, horse stable, barnyard and elastoplast aromas (Lonvaud-Funel, 1999). These aroma descriptors are generally associated with the presence of the spoilage yeast *Brettanomyces* (Chatonnet *et al.*, 1992). The ability of LAB to produce volatile phenols supports the theory that these characteristic aromas are not solely produced by *Brettanomyces*. This theory was supported by results from Nelson (2008). In this study, the influence of different MLF scenarios on the production of volatile phenols was investigated. Lactic acid bacteria used in this study were able to produce significant levels of volatile phenols. It was also found that spontaneous MLF resulted in higher levels of volatile phenols.

TABLE 7

A summary of routinely used methods for malic acid and MLF monitoring including the advantages and disadvantages of each method [compiled from Kollar & Brown (2006) and Theodore (2006)].

Monitoring Technique	Advantages	Disadvantages
Paper Chromatography (PC) – separate compounds based on their polarity – visually follow disappearance of malic acid – commonly used in winery	– easy to use – simple, affordable and indicative of MLF progress	– strictly qualitative so still need quantitative values to verify MLF completion – not precise – not specific for L–malic acid
Thin Layer Chromatography (TLC) – similar to PC but uses TLC plates instead of paper	– easy to use – simple and affordable – results in one hour; much faster than PC	– not precise – not specific for L–malic acid; – strictly qualitative so still need quantitative values to verify MLF completion
Reflectance – Reflectoquant® – based on reflectance photometry – use reactive test strips to analyze for various wine components	– a fraction of the cost of a spectrophotometer – half of the cost of an enzymatic kit – measure multiple wine parameters – fastest method currently available (5 min/sample) – relative accuracy of 10%	– measure relative malic acid levels so still need to qualify absolute levels – operating range 1 to 60 mg/L, so some samples need to be diluted or decolourised – need to be calibrated with reference method
Enzymatic analysis – uses enzyme that specifically react with L–malic acid then use UV–visible spectrophotometer to monitor enzymatic reaction – most commonly used method – MLF complete if malic acid is less than 200 to 300 mg/L	– quantitative – excellent precision – kits readily available – quantify very low levels of malic acid – results in 30 minutes	– more complex – more expensive – short shelf life of reagents after activation – require use of accurate micro–pipettes – turbid samples need to be centrifuged
Capillary electrophoresis (CE)	– highly accurate – short analyses time, fast results	– extremely expensive – not recommended for everyday use in winery
Fourier–Transform Infrared (FT–IR) Spectroscopy – use infrared spectra to quantify wine parameters	– accurate – small sample volume – short analyses time, fast results	– expensive equipment – accuracy dependant on reference values and calibration curve
High performance liquid chromatography (HPLC) – separation of compounds based on polarity and interaction with stationary or solid phase	– highly accurate	– extremely expensive – not recommended for everyday use in winery

Despite the fact that it has been established that LAB contribute to the volatile phenol concentration, it is still unclear if strains of *O. oeni* are able to produce levels of 4-vinylguaiacol and 4-vinylphenol that could be of sensorial significance (Swiegers *et al.*, 2005). This was supported by the findings of Gámbaro *et al.* (2001), which only saw a small increase in 4-vinylguaiacol and 4-vinylphenol in Tannat wines that underwent MLF. These levels were below the odour threshold.

The fact that LAB are able to contribute to the volatile phenol concentration in wine, emphasise the need to screen commercial MLF cultures for the potential to produce volatile phenols.

Acetic acid

Acetic acid is the most important volatile acid produced during fermentation, both quantitatively and sensorially. The flavour threshold for acetic acid is dependent on both the type and style of wine (Bartowsky & Henschke, 1995; Lonvaud-Funel, 1999; Ugliano & Henschke, 2008). Acetic acid leads to a sour, pungent and vinegar aroma in wine (Francis & Newton, 2005) in concentrations exceeding 0.7 g/L (Swiegers *et al.*, 2005). Lower concentrations, 0.2 to 0.6 g/L, can contribute to the complexity of wine aroma. There is an increase in acetic acid of 0.1 to 0.2 g/L, which is generally associated with MLF (Bartowsky & Henschke, 1995).

There are two proposed mechanisms by which LAB can increase acetic acid levels in wine. If MLF commences before the completion of AF, the LAB are able to ferment hexoses that have not been completely fermented by the yeast. *Oenococcus oeni* is a heterofermentative strain and will not only produce ethanol and CO_2, but also acetic acid and D-lactic acid, via the 6-PG/

TABLE 8

A summary of molecular techniques available for monitoring and characterisation of the microbial population during MLF.

Technique	Application	Reference
Polymerase chain reaction (PCR)	- Specific PCR primers target and amplify either 16S rRNA genes or genes encoding the MLE - distinguish LAB genera	Bartowsky *et al.* (2003) Zapparoli *et al.* (1998)
Randomly amplified polymorphic DNA (RAPD) analysis (a PCR-based technique)	- Quick and sensitive discrimination of LAB strains - Follow *O. oeni* population changes during MLF	Bartowsky *et al.* (2003)
PCR-denaturing gradient gel electrophoresis (PCR-DGGE)	- Identify and distinguish LAB - Monitor spoilage microorganisms during fermentation	Renouf *et al.* (2006) Spano *et al.* (2007)
Real-time PCR and differential real-time PCR assay	- Rapid detection and quantification of *O. oeni* - Enumerate total LAB population to assess spoilage risk of juice/wine by LAB	Pinzani *et al.* (2004) Neeley *et al.* (2005)
Restriction analysis of the amplified 16S-rDNA (PCR-ARDRA)	- Identification of species of LAB	Rodas *et al.* (2003)
Transverse alternating field lectrophoresis (TAFE) and Pulsed-field gel lectrophoresis (PFGE)	- Patterns of digested chromosomal DNA used to differentiate closely related *O. oeni* strains	Versari *et al.* (1999)
Contour-clamped homogenous electric field (CHEF) (a specific type of PFGE)	- Most reliable for strain differentiation - Produce unique DNA fingerprint for individual strains	Bou & Powell (2006)

PK pathway. As a consequence, the volatile acidity increases slightly (Lonvaud-Funel, 1999; Swiegers *et al.*, 2005). During the formation of diacetyl, LAB can also produce acetic acid during the first reaction of the citric acid metabolic pathway catalysed by the citrate lyase enzyme (Bartowsky *et al.*, 2002b; Bartowsky & Henschke, 2004). The rate of acetic acid accumulation is dependent on the rate of MLF, with higher concentrations of acetic acid being formed in conjunction with a higher MLF rate (Lonvaud-Funel, 1999). It has been shown by recent studies that the common belief that co-inoculation will lead to higher acetic acid concentrations is not true.

Volatile fatty acids

Volatile fatty acids are formed by the hydrolysis of tri-, di- and monoacylglycerols (lipids) (Liu, 2002). Wine consists of a mixture of straight chain fatty acids and branched chain fatty acids. The straight chain fatty acids are usually referred to as short chain (C_2-C_4), medium chain (C_6-C_{10}) or long chain (C_{12}-C_{18}) fatty acids (Ugliano & Henschke, 2008). As the chain length of fatty acids increase, the volatility decreases and the odour changes from sour to rancid and cheesy (Francis & Newton, 2005; Ugliano & Henschke, 2008).

Maicas *et al.* (1999) found no significant increase in isovaleric, isobutyric and hexanoic acids after MLF, although capric acid and caprylic acid levels were higher. This lack of significant increase could be beneficial to wine aroma due to the fact that isobyturic and isovaleric acids are associated with rancid, butter, cheese and sweaty aromas (Francis & Newton, 2005). Similarly, Herjavec *et al.* (2001) saw a significant increase in caprylic acid, as well as increased in caproic- and capric acid concentrations. Lipases are able to produce volatile fatty acids but the lipase activity in wine LAB still warrants further investigation (Liu, 2002).

Higher alcohols

Higher alcohols are formed by the decarboxylation and subsequent reduction of α-keto acids. The keto acids are produced as intermediates during amino acid biosynthesis and catabolism, the latter referred to as the Ehrlich pathway. Amino acid biosynthesis is responsible for most of the higher alcohols formed during fermentation (Ugliano & Henschke, 2008). At lower concentrations (less than 300 mg/L), higher alcohols can contribute to the complexity and fruity aromas in wine, whereas higher concentrations (above 400 mg/L) could be detrimental to wine aroma and quality due to the harsh chemical-like aromas (Swiegers *et al.*, 2005).

Jeromel *et al.* (2008) found that MLF had an insignificant effect on the higher alcohol concentration of wine, except for significant increases in isobutanol and 2-phenylethanol. In contrast, Herjavec *et al.* (2001) found no change in levels of 1-propanol, isobutanol, isoamyl alcohol or 2-phenylethanol. This is supported by Maicas *et al.* (1999) who found the production of isobutanol, 1-propanol, 1-butanol and isoamyl alcohol to be dependent on the strain used to perform MLF. Pozo-Bayón *et al.* (2005) saw increased levels of higher alcohols after MLF, but none of the increases were significant. The fact that LAB seem to have limited ability to produce higher alcohols could be beneficial, as most of these compounds impart harsh solvent-like aromas in the wine. The concentration of higher alcohols that have either a positive or

negative influence on the wine aroma, is likely to depend on both the aroma intensity of the respective alcohols as well as the style of wine (Ugliano & Henschke, 2008).

Based on the available literature, it is clear that MLF has an effect on the sensory character of wine. These effects are diverse and sometimes contradicting and may be due to the following factors: the influence of the different bacteria strains, the presence and availability of precursors, LAB associated enzymatic activity, the wine type as well as the intensity of the inherent wine flavour and cultivar character, the vinification conditions under which the wine was produced as well as the training and skills of the sensory panel that evaluate the wine.

Malolactic fermentation generally leads to an increase in the buttery attribute, reduced vegetative character, modification in the fruitiness and improved mouthfeel and flavour persistence. Wine aroma is also influenced by the type of LAB and possible wood interactions. Due to the influence that MLF has on the aroma properties of a wine, it is essential for the winemaker to understand the formation of these compounds, the factors that influence their occurrence in wine and the ways in which to manipulate their production. This will enable wine producers to create a specific style of wine in an industry where consumer preference is the driving force for product development.

Future research should include the investigation into the identification and quantification of relevant aroma precursors; the vineyard practices that influence their occurrence and concentration, the effect of assorted vinification processes on the evolution of these precursors to aroma active compounds (Swiegers *et al.*, 2005), as well as the mechanisms of how LAB contribute to this process. The enzymatic profiles of wine LAB warrant further investigation as well as the factors that influence the activity of these enzymes under winemaking conditions. The choice of bacterial strain seems to be one of the most influential factors on the production of odour-impact compounds associated with MLF.

IMPACT OF MALOLACTIC FERMENTATION ON WINE WHOLESOMENESS

Biogenic amines

Biogenic amines are a group of organic nitrogen-containing compounds. The main biogenic amines associated with wine are putrescine, histamine, tyramine and cadaverine, followed by phenylethylamine, spermidine, spermine, agmatine and tryptamine (Ten Brink *et al.*, 1990; Lonvaud-Funel, 2001). The role of biogenic amines in wine and the microorganisms involved in their synthesis, were recently reviewed by Smit *et al.* (2008).

Biogenic amines are formed by certain LAB via the substrate-specific enzymatic decarboxylation of naturally occurring amino acids (Ten Brink *et al.*, 1990; Lonvaud-Funel, 2001). These compounds are of importance in wine due to their potential toxicological effects in sensitive humans. These include symptoms like headaches, hypo- or hypertension, cardiac palpitations and in extreme cases even anaphylactic shock (Shalaby, 1996). It is imperative to be able to identify strains with the potential to produce biogenic amines. The ingestion of biogenic amines, histamine in particular, can lead to various health reactions in sensitive humans. Phenylethylamine and tyramine can cause symptoms of high blood pressure and migraines. Putrescine and cadaverine, besides being able to enhance the toxicity of histamine, tyramine and phenylethylamine, can also have a detrimental effect on wine quality by imparting flavours of putrefaction and rotten meat, respectively (Shalaby, 1996; Palacois, 2006). The presence of alcohol, SO_2 and other amines could potentially amplify the toxic effect of certain biogenic amines (Fernandes & Ferreira, 2000; Volschenk *et al.*, 2006). There are various factors that influence the biogenic amine content. These factors include the amino acid composition, the microflora present in the wine and the ability of the microflora to decarboxylate amino acids. All parameters that favour bacterial growth will favour biogenic amine formation (Lonvaud-Funel & Joyeux, 1994; Volschenk *et al.*, 2006).

The essential role of LAB and MLF in the formation of biogenic amines has been confirmed by various authors (Lonvaud-Funel & Joyeux, 1994; Moreno-Arribas *et al.*, 2000; Marcobal *et al.*, 2006; Volschenk *et al.*, 2006; Landete *et al.*, 2007a). Lonvaud-Funel & Joyeux (1994) reported increased concentrations of biogenic amines after MLF and Landete *et al.* (2007a) reported histamine, tyramine, phenylethylamine and putrescine production by LAB. Similarly, in a study on the changes in biogenic amine concentration during the industrial manufacturing of red wines, Marcobal *et al.* (2006) identified MLF as the main mechanism of biogenic amine formation, especially the production of histamine, tyramine and putrescine.

It is generally accepted that spoilage LAB are responsible for the formation of biogenic amines, specifically species of *Pediococcus* and *Lactobacillus* (Moreno-Arribas & Polo, 2008). Landete *et al.* (2007b) identified *L. brevis* to be the main producer of tyramine and phenylethylamine. Arena & Manca de Nadra (2001), as well as Manfroi *et al.* (2009), highlighted the ability of *L. hilgardii* to produce putrescine and also found that *L. plantarum* strains have the ability to produce biogenic amines. Recent research also identified *O. oeni* as a possible biogenic amine producer. Moreno-Arribas *et al.* (2000) identified *O. oeni* as the main LAB responsible for histamine formation and lactobacilli for tyramine formation. Lucas *et al.* (2008) identified 54 colonies of histamine producing isolates as *O. oeni* and despite the fact that histamine producing *O. oeni* are frequently found in wine, it was also found that LAB may lose this ability due to instability of the phenotype. Histamine producing LAB all carry an *hdcA* gene coding for a histidine decarboxylase (HDC) that converts histidine to histamine. This *hdcA* gene was detected on a large and possibly unstable plasmid, which could result in a loss of histamine producing ability.

In an investigation of the biogenic amine producing capability of several strains of *O. oeni*, more than 60% were able to produce histamine in concentrations ranging from 1.0 to 33 mg/L. An additional 16% had the added capability of producing putrescine and cadaverine (Geurrini *et al.*, 2002). Landete *et al.* (2005a) showed the highest frequency of histamine production by *O. oeni*. In the same study, *O. oeni* was also shown to produce the lowest concentrations of histamine, whereas higher concentrations were produced by *Lactobacillus* and *Pediococcus* strains, specifically *P. parvulus* and *L. hilgardii*. In contrast, Izquierdo *et al.* (2009) found that *O. oeni* did not significantly contribute to the overall biogenic amine content in wine. Rosi *et al.* (2009) studied 26 strains of *O. oeni* for their biogenic amine formation ability in synthetic medium and wine. These authors found that the concentration of histamine and tyramine formed by *O. oeni* were

dependant on the bacterial strain, the effect of the yeast strain on the wine composition, the length of bacteria-yeast contact time after MLF completion, as well as the screening method used for biogenic amine determination.

There are various oenological parameters that influence the decarboxylase enzyme activity as well as the biogenic amine producing ability of LAB (Landete *et al.* 2008). Histidine decarboxylase activity is enhanced at pH 3.5 and has an optimum pH of 4.8 (Lonvaud-Funel & Joyeux, 1994). Tyrosine decarboxylase (TDC) is active in the pH range of 3 to 7, but exhibits optimum activity at pH 5 (Moreno-Arribas & Lonvaud-Funel, 1999). In wines with higher pH values, decarboxylase positive bacteria are more likely to survive. This means that in most cases, a higher pH will concomitantly lead to higher biogenic amine concentrations (Wibowo *et al.*, 1985; Lonvaud-Funel & Joyeux, 1994; Gardini *et al.*, 2005; Landete *et al.*, 2005b; Martin-Álvarez *et al.*, 2006). At a higher pH, the SO_2 fraction will be less effective which can also result in a higher concentration of biogenic amines (Gerbaux & Monamy, 2000). A higher SO_2 concentration will also prevent the formation of biogenic amines by reducing the viable LAB population in wine (Marcobal *et al.*, 2006). Another important factor is the ethanol content of the wine. In general, higher ethanol concentrations lead to a decrease in the formation of biogenic amines (Gardini *et al.*, 2005). It was found that a high ethanol concentration reduces HDC activity by altering the membrane properties of LAB and thereby slowing down histidine transport (Rollan *et al.*, 1995). Lonvaud-Funel & Joyeux (1994) found that an ethanol level of up to 10% (v/v) enhances HDC activity and Mazzoli *et al.* (2009) saw a decrease in bacterial growth and biogenic amine formation at ethanol concentrations exceeding 13% (v/v).

The ability to produce biogenic amines is used as a screening criterion in the selection of LAB starter cultures. Le Jeune *et al.* (1995) developed a detection system for histamine producing LAB strains and more recently, Marcobal *et al.* (2005) selected three primer pairs to use in a multiplex-PCR assay to simultaneously detect histamine, tyramine and putrescine producing LAB. The assay yielded a 367 bp DNA fragment from histidine decarboxylases (*hdc*) (primer pair JV16HC/JV17HC), a 924 bp fragment from tyrosine decarboxylases (*tdc*) (primer pair P1-rev/P2-for) and a 1446 bp fragment from ornithine decarboxylases (*odc*) (primer pair 3/16). The first PCR detection for cadaverine producing LAB has also been developed (De las Rivas *et al.*, 2006).

In a study investigating the potential of commercial cultures to produce tyramine, histamine and putrescine, it was found that none of the commercial starter cultures produced biogenic amines (Moreno-Arribas *et al.*, 2003). In a study comparing spontaneous and inoculated MLF in Spanish red wine, the incidence of biogenic amines was reduced in the inoculated MLF (Martín-Álvarez *et al.*, 2006). Similarly, Izquierdo *et al.* (2007) determined that histamine, tyramine and putrescine concentrations increased by 68% in Spanish wines due to spontaneous MLF.

Inoculation for MLF with a starter culture that does not have the ability to produce biogenic amines will eliminate the risk of biogenic amine formation associated with spontaneous MLF.

Ethylcarbamate

Ethylcarbamate (EC) is a suspected carcinogen (Fugelsang & Edwards, 1997). Lactic acid bacteria, including commercial strains of *O. oeni*, are able to degrade arginine via the arginine deiminase pathway. There are three enzymes that play a role in this pathway. Arginine deiminase is responsible for the production of L-citrulline from L-arginine. Ornithine transcarbamylase then converts L-citrulline to L-ornithine and carbamyl phosphate. The final reaction is catalysed by carbamate kinase during which ATP is generated from carbamyl phosphate. The catabolism of arginine contributes to LAB growth due to the generation of ATP, but two of the intermediates formed, citrulline and carbamyl phosphate, are able to react with ethanol to form EC (Liu *et al.*, 1994, 1995; Arena & Manca de Nadra, 2002; Volschenk *et al.*, 2006; Araque *et al.*, 2009). Strains of *O. oeni* and *Lactobacillus buchneri* are able to excrete citrulline and carbamyl phosphate (Liu *et al.*, 1994; Mira de Orduña *et al.*, 2000, 2001) and Uthurry *et al.* (2006) also found that strains of *O. oeni* and *L. hilgardii* were able to contribute to the EC concentration. Recently, Romero *et al.* (2009) found *L. plantarum* strains in this study were unable to degrade arginine to form citrulline.

Araque *et al.* (2009) investigated the presence of genes involved in the deiminase pathway that are responsible for the degradation of arginine in different LAB species. The degrading strains included *L. brevis* and *L. hilgardii*, *O. oeni*, *P. pentosaceus*, and some strains of *Leuc. mesenteroides* and, contrary to Romero *et al.* (2009), also *L. plantarum*. Uthurry *et al.* (2006) also found increased concentrations of EC after MLF in Tempranillo and Cabernet Sauvignon wines, irrespective of the bacterial strain or different conditions of pH and temperature. In contrast, Romero *et al.* (2009) found the conditions that led to a slight increase in EC formation by *O. oeni* to be high ethanol concentrations, low pH, high L-malic acid concentrations and higher temperatures.

Inhibition of the LAB population immediately after the completion of MLF could avoid the formation of citrulline from arginine and concomitant EC formation (Terrade & Mira de Orduña, 2006).

MALOLACTIC FERMENTATION MONITORING

Monitoring of malic acid concentration

The decrease in malic acid or increase in lactic acid is mostly used to monitor the progression of MLF. There are various useful analytical techniques available for monitoring the malic acid concentration. These methods include chromatography, reflectance and enzymatic assays, as well as analytical techniques like Fourier-transform infrared (FT-IR) spectroscopy and capillary electrophoresis (CE) or the use of high-performance liquid chromatography (HPLC). These techniques differ in their accuracy, time needed for analysis as well as the cost involved. A summary of the monitoring techniques, as well as the advantages and disadvantages, are provided in Table 7.

Chromatography, like paper chromatography (PC) and thin layer chromatography (TLC), is the method most often implemented in wineries due to the low cost involved. Unfortunately, these methods are not as accurate as some of the analytical techniques. The more accurate methods usually involve the acquirement of expensive equipment like a CE or HPLC. In order to accurately monitor the progression of MLF, fast and accurate results are required. The use of an enzymatic kit could address both of these aspects. Although the cost involved is still relatively high, it is

still less expensive than acquiring instruments like an HPLC or a FT-IR spectrometer. The commercial scale of the cellar and the amount of samples to be analysed on a regular basis, will greatly influence the selection of the most suitable malic acid monitoring technique.

Monitoring of microbial population

Monitoring of the microbial population is important in identifying the LAB responsible for MLF, possible spoilage LAB as well as determining the viable microbial population. This will provide the winemaker with control over the MLF process, as well as preventing possible problems before they arise. There are two established microbiological techniques that are generally used, including microbial plate counts and microscopy.

Microbial plate counts refer to the isolation of LAB after which the number of viable LAB in the wine is determined. This requires the growth of the bacterial cells on a nutrient medium. An advantage of this method is the fact that spoilage LAB like *Pediococcus* and *Lactobacillus* can grow in 2 to 4 days, so results can be quickly obtained. On the other hand, the slow growth of *O. oeni*, up to 7 days, can mean a delay in obtaining the results. This method also requires appropriate sterile equipment and nutrient media.

Microscopy is an alternative technique for monitoring the microbial population and is based on the direct observation of a wine sample using a microscope. This allows for fast evaluation of the microflora in the wine. It is possible to instantly identify the bacterial population due to the distinct morphologies which allow for discrimination of wine LAB (Kollar & Brown, 2006). *Oenococcus oeni* are some of the smallest cells in wine and appear round or slightly elongated and usually form distinct chains of individually linked cells. It is generally accepted that the longer the chains, the 'healthier' the population. If only single cells or pairs of *O. oeni* are visible (except directly after starter culture additions when chains are broken because of the drying process), the culture is usually no longer viable. *Pediococcus* cells are almost completely round and do not form chains. They appear singly, in pairs, tetrads or small bunches and appear bright white under the microscope. *Lactobacillus* is rod shaped and appears as single cells or pairs in wine and also appears bright white under the microscope (Dicks & Endo, 2009). The disadvantage of this technique is the fact that it requires a quality bright field microscope with 1000X magnification capability. This method is also not quantitative without specific tools (Kollar & Brown, 2006).

There are various molecular techniques available that aid in the characterisation of LAB and add to the knowledge of these bacteria and their role in the winemaking process (Lonvaud-Funel, 1995). These techniques enable us to identify microbes, differentiate LAB from each other as well as distinguish between different strains within the same species (Table 8) (Bartowsky *et al.*, 2003). Some of these techniques include: DNA-DNA hybridisation, 16S and 23S rRNA sequence analysis, DNA-fingerprinting and pulse-field gel electrophoresis (PFGE) as well as PCR-based DNA fingerprinting known as randomly amplified polymorphic DNA (RAPD) analysis (Bartowsky *et al.*, 2003). These techniques are used to identify and differentiate between LAB (Zapparoli *et al.*, 1998; Bartowsky & Henschke, 1999).

Despite the fact the PFGE analysis is the most reliable technique for strain differentiation, as well as being used in strain selection for new starter cultures, it takes up to 3 days to generate results. Future techniques that require further development and need to be improved, include DNA sequencing, amplified fragment length polymorphism (AFLP), ribotyping as well as species-specific and multiplex-PCR.

CONCLUSIONS

The information available on MLF and LAB can assist the winemaker in ensuring successful MLF which involves the complete degradation of malic acid, generating a microbiologically stable wine as well as ensuring a positive aroma contribution by the LAB.

Inoculation with a commercial starter culture will reduce the risks associated with spontaneous MLF. These cultures are selected for their ability to survive in the challenging wine environment and to successfully carry out MLF. Co-inoculation is a strategy with the potential to reduce the duration of MLF and risks associated with after AF inoculation, as well as contributing positive aroma properties to the wine without the excessive production of acetic acid.

The physiochemical parameters that the winemaker can control include the temperature, pH and SO_2 additions. Maintaining temperatures of 18 to 22°C, a pH of 3.2 to 3.4 and total SO_2 concentrations of below 30 mg/L, will optimise conditions for bacterial survival and proliferation. Besides these parameters, a crucial decision by the winemaker involves the selection of the yeast strain to perform AF and the bacteria strain selected for MLF. This selection is an important consideration to ensure minimal antagonistic interactions between the yeast and bacteria that could be detrimental to both the execution of AF and MLF. The yeast strain should produce low amounts of possible inhibitory compounds like SO_2 and medium chain fatty acids. The ability of LAB to survive in the wine environment and withstand the effects of these inhibitory compounds is unequivocally strain dependant.

It has been proven that MLF has a significant impact on the final wine aroma profile. There are various aroma compounds, imparting negative and positive characteristics to the wine, which are produced by the LAB. Factors that influence the production of these compounds need to be investigated. This will provide an invaluable tool in the production of a certain type and style of wine. The production of certain aroma compounds are not just strain dependant, but also differ between the LAB genera. In order to capitalise on these differences, novel approaches for the development of starter cultures are needed. Different genera of LAB as well as a mixture of LAB cultures could be considered for future use in starter cultures.

The continuous monitoring of MLF is essential and often neglected by winemakers. This allows the winemaker to follow the progression of malic acid degradation as well as the bacteria responsible for the fermentation. This is also a way for the winemaker to identify possible difficulties before they can affect the quality of the wine.

Successful MLF is a process that requires specific bacterial strain selection, particular physiochemical parameters and constant monitoring.

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