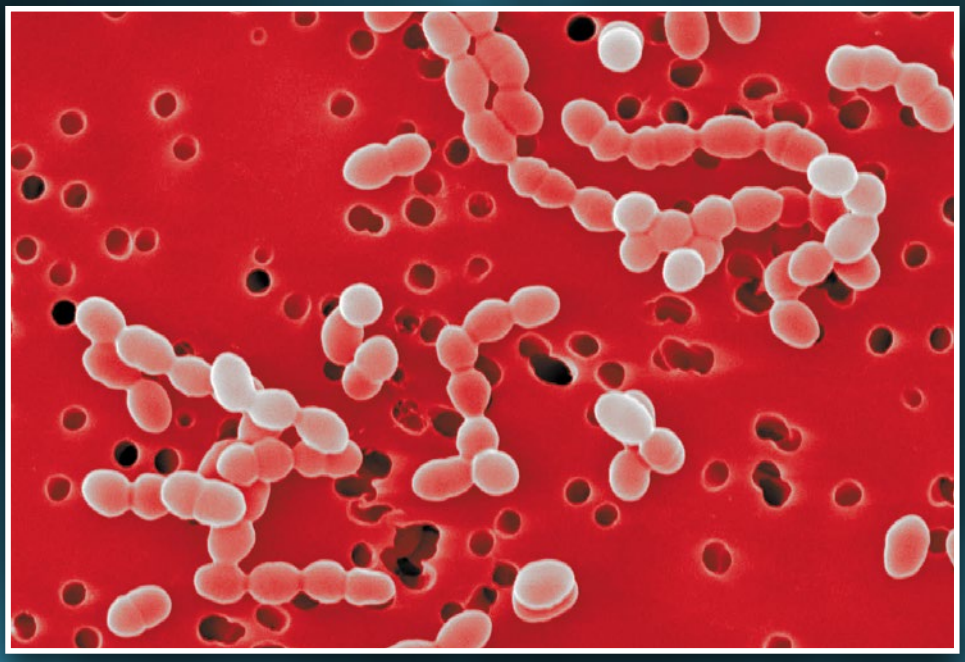
MALOLACTIC FERMENTATION- IMPORTANCE OF WINE LACTIC ACID BACTERIA IN WINEMAKING



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MALOLACTIC FERMENTATION-
IMPORTANCE OF
WINE LACTIC ACID BACTERIA
IN WINEMAKING

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FACTORS INFLUENCING THE SURVIVAL AND GROWTH OF LACTIC ACID BACTERIA

The survival and growth of LAB in wine can be influenced by the following factors:

- The chemical and physical composition of the wine
- Factors associated with vinification
- The interaction between LAB and other micro-organisms

Wine pH exerts a strong selective action that largely determines which strain(s) of LAB will be present. It influences their viability, rate of growth, speed of L-malic acid degradation and metabolic behaviour. A wine pH of 3.5 is pivotal because below this value wine microflora are easier to control. Although MLF is more difficult to induce at lower pH levels, under these conditions wine LAB will conduct MLF mostly with no deleterious effects on wine quality. SO_2 strongly inhibits the growth of LAB, but the sensitivity of LAB to SO_2 varies, and SO_2 is more inhibitory at low pH. Both the growth of LAB and the progress of MLF are increasingly inhibited as alcohol concentrations rise above 6%, with 14.5% v/v the upper limit tolerated by most MLB. Wine LAB are mesophilic, with an optimal growth temperature between 15° and 30°C (59° and 86°F). The rate of bacterial growth and speed of MLF are strongly inhibited by lower temperatures.

Certain winery practices, such as juice and wine clarification, can remove a large portion of the LAB. This will reduce bacterial growth and its resulting effect on wine quality (Henick-Kling 1988). During clarification, some nutrients and suspended particles that are stimulatory to bacteria growth will be removed. Wines made by thermo-vinification have been reported as being less suitable for MLF. The timing of the inoculation with MLB influences the kinetics of MLF.

With respect to interactions with other wine organisms, the presence of indigenous yeast and/or bacterial microflora, along with the selected yeast used to conduct the AF, may introduce the possibility of an antagonistic effect and/or synergistic relationships. In some minor cases, however, there may be no observable effect. In winemaking, interactions between MLB and yeasts, other fungi, acetic acid bacteria, bacteriophages or other LAB are known (Ribéreau-Gayon et al. 2000). The antagonistic effect attributed to yeast has been explained by nutrient competition, SO_2 production or the presence of medium-chain fatty acids, all of which are capable of inhibiting MLB growth. Conversely, yeast may favour the growth of LAB and stimulate the MLF. During extended lees contact with wine, the process of yeast autolysis releases vitamins and amino acids. This results in nutrient enrichment and subsequent

stimulation of the MLF. Costello et al. (1985) reported that growth of *Pediococcus* sp. was supported by the rapid cell death of *O. oeni,* and under high pH conditions the early growth of *L. brevis* will completely inhibit the growth of *O. oeni*. Recently, Gerbaux (in a personal communication) showed that wine conditions that stimulate MLF will inhibit the development of *Brettanomyces* spoilage yeast (Gerbaux et al. 2009).

THE INFLUENCE OF MALOLACTIC FERMENTATION ON WINE COMPOSITION

MLF is not only the simple decarboxylation of L-malic acid to L-lactic acid and CO_2. Wine components are consumed by wine LAB, which means they will produce metabolic end products from those components. This has an influence on grape-derived aroma compounds and on those arising from the AF, and confers biological stability on the final product (Davis et al. 1985). The growth of MLB is generally encouraged where MLF is required to reduce the acidity of the wine. The reduction of acidity is beneficial to the quality of wines made in cool winegrowing regions, because the grapes naturally contain high levels of organic acids. Worldwide consumer preference currently favours fruit-driven wines with moderate acidity, resulting in acid reduction becoming a critical issue with wines produced in cool climates. This, coupled with positive flavour changes associated with growth of MLB in wine, has made MLF a desirable process for almost all red wines and for certain styles of white wines. Growth of LAB in wine must be controlled to ensure desirable MLB that produce no off-flavours. In most cases, MLF should complete rapidly to save processing time and achieve early stability of the product. In no instance should indigenous strains of LAB be relied on to conduct the MLF.

Apart from producing lactic acid as a major end product of sugar catabolism (Henick-Kling 1993), LAB are known to produce other flavour-active compounds, such as acetaldehyde, acetic acid, diacetyl, acetoin and 2,3-butanediol. Diacetyl, acetoin and 2,3-butanediol mainly originate from the bacterial consumption of citric acid, and are of considerable importance to the flavour profile of wine. In lower concentrations, these compounds are thought to add complexity to flavour. At concentrations in excess of 5 mg/L, diacetyl can be overpowering, resulting in distinct buttery/nutty flavours. Depending on the pH and the oxidation-reduction potential, acetic acid can be another product of citric acid metabolism by wine LAB. Increased levels of alcohol, volatile esters and ethyl lactate were reported in wines undergoing MLF (Meunier and Bott 1979). Henick-Kling et al. (1992) described flavour contributions attributed to individual strains of MLB. Recently, numerous studies have been conducted on the impact of MLF on the wine sensory profile. Bartowsky et al. (2011) described effects on red berry fruity characters of red wine attributed to strain-specific influences by MLB. Sumby et al.

(2009) reported intracellular esterase activity in *O. oeni*. Christen et al. (2011) studied the metabolism of free and SO_2-bound acetaldehyde by wine LAB. The duration of MLF in Riesling wine was strain-dependent (figure 2). Except for four strains, all *O. oeni* strains depleted malic acid within 17 to 22 days. The other four strains required 27 to 45 days to complete malic acid degradation, and the control did not undergo MLF. The degradation of acetaldehyde was completed 2 to 10 days after the degradation of malic acid. The authors concluded wine LAB play an essential role in acetaldehyde removal after the AF completes, and proposed delaying the SO_2 addition until one week after L-malic acid disappeared. It was thought that, by so doing, bound-SO_2 levels would be reduced by up to 75%.

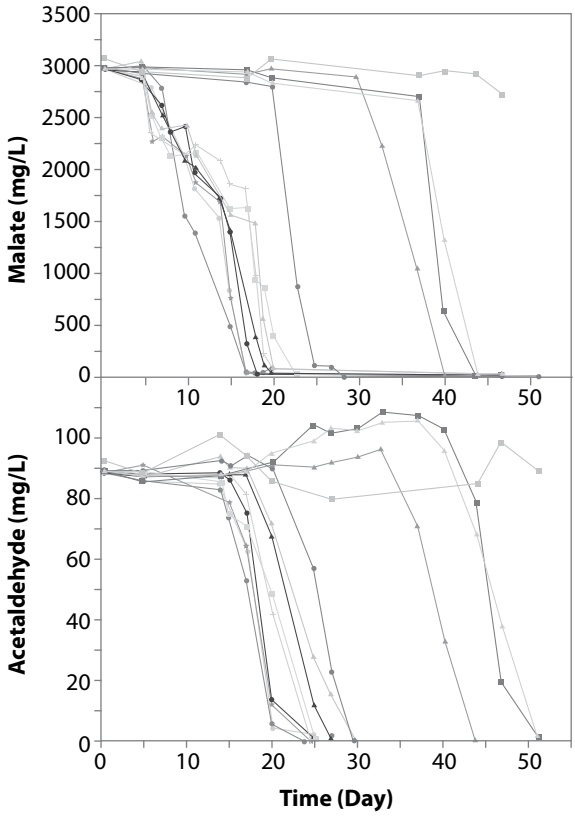

Figure 2. Degradation of malic acid (top graph) and acetaldehyde (bottom graph) by 12 strains of commercial wine lactic acid bacteria in a Riesling wine

Flamini et al. (2002) attributed lessening of vegetal notes to degradation of ethanal and higher aldehydes such as hexanal and heptanal by LAB during MLF.

SPOILAGE BY LACTIC ACID BACTERIA

MLF is not always beneficial and can be responsible for undesirable changes to the sensory properties of wine. As previously mentioned, several species of LAB may conduct MLF. MLF that occurs at a pH below 3.5 and is induced by catalogued, commercial strains is generally conducted by *O. oeni,* which is less likely to generate off-odours than indigenous MLB strains. Excessive amounts of acetic acid, as well as buttery, cheesy, milky, metallic or earthy odours, are usually present when indigenous strains of *Pediococcus* sp. and/or *Lactobacillus* sp. catalyze MLF at pH levels in excess of 3.5.

LAB can produce biogenic amines by the decarboxylation of amino acids. Histamine, derived from the decarboxylation of histidine, is felt to cause a reaction in sensitive individuals if the wine contains more than 0.1 mg/L. Of the LAB, *Pediococcus* sp. and heterofermentative *Lactobacillus brevis* are regarded as the two biggest producers of histamine (Lonvaud-Funel 2001). Both these genera are usually found in wines with a pH above 3.5; consequently biogenic amine synthesis appears to be more prevalent in wines exhibiting a high pH. If bacterial strains capable of producing biogenic amines are known to be present, the winemaker should inoculate the wine with a selected malolactic starter culture capable of replacing the indigenous LAB. Bacteria have a slight capacity to form histamine only during their active growth phase. However, it has been shown (Lonvaud-Funel 2001) that a non-proliferating bacterial flora can develop considerable amounts of histamine. Therefore, the bacterial population of a wine should be eliminated by SO_2 addition followed by clarification as soon as the MLF has completed; this is especially important in high pH wines. Other undesired sensory changes, due to the metabolism of indigenous LAB, include mousy taints, colour changes and ropiness.

THE CONTROL OF MALOLACTIC FERMENTATION

Prevention

If MLF is not desired, the growth of LAB in grape must or wine should be suppressed by removing or inactivating the bacteria present. Although MLF is occasionally difficult to induce, the prevention of LAB can also be difficult. The addition of 50 to 100 mg/L of SO_2 to the must, depending on the pH, destroys more than 90% of the viable bacteria present. The effect of SO_2 is dependent on wine pH, with SO_2 being more effective at lower pH levels. In high pH wines, a combination of SO_2 and lysozyme, or lysozyme alone, should be considered. Inhibition of MLF can be accomplished by the following conditions… *(Complete text available in printed version of the book.)*

THE CHEMISTRY OF MALOLACTIC FERMENTATION

PETER COSTELLO, PH.D.

The term "malolactic fermentation" (MLF) describes the enzymatic conversion of L-malic acid to L-lactic acid and CO_2 by lactic acid bacteria (LAB) cells after they have grown (Wibowo et al. 1985). This biological deacidification reaction is well recognized as one of the main metabolic capabilities of LAB in wine, and its conduct is of major commercial importance to the winemaking process. In addition to the deacidification reaction that characterizes MLF, it is becoming increasingly recognized that a diverse range of other metabolic activities are associated with the growth and development of LAB in wine, which can have a significant influence on wine quality. In this section, the primary metabolic characteristics of LAB are outlined, with particular emphasis given to MLF, as well as to some of the other metabolic reactions of LAB that can affect wine properties.

GENERAL METABOLIC PROPERTIES OF LACTIC ACID BACTERIA

LAB constitute a ubiquitous group of bacteria that occur in a range of environments, including many foods and beverages. Importantly, these bacteria are primarily noted for their ability to produce lactic acid from a fermentable carbohydrate source. Lacking heme-linked cytochromes and catalase, LAB obtain energy from carbohydrates by fermentative metabolism (Kandler 1983).

LAB can be broadly classified as either homofermentative or heterofermentative according to the types of end products produced from the fermentation of glucose. Homofermentative LAB, including the *Pediococci* and some of the *Lactobacilli*, utilize the Embden-Meyerhof-Parnas (EMP) glycolytic pathway to convert the hexose sugar – glucose – mainly to lactic acid. In this pathway, two moles of lactic acid and two moles of adenosine triphosphate (ATP) are produced for each mole of glucose fermented. On the other hand, the heterofermentative *Lactobacilli* and the *Leuconostocs* lack some key enzymes of the EMP pathway and ferment

hexose sugars by the phosphoketolase pathway. In this pathway, equimolar concentrations of lactic acid, CO_2 and acetic acid or ethanol can be produced from one mole of glucose, with a concomitant energy gain of one mole of ATP. The oxidation/reduction potential (redox) of the system also affects the ratio of ethanol/acetic acid produced, with aerobic conditions favouring the formation of acetic acid, and anaerobic conditions favouring the production of ethanol (Kandler 1983, and Condon 1987). Depending on the species or the genus of LAB involved, the isomers of lactic acid produced from the fermentation of carbohydrates can be either L(+), D(-) or a combination of both the L(+) and D(-) forms (Kandler 1983, Boulton et al. and Condon 1998). For example, *Leuconostocs*, including *Oenococcus oeni,* produce the D(-)-lactic acid isomer from the fermentation of hexose sugars. In contrast, however, the decarboxylation of L(-)-malic acid in the malolactic fermentation yields only the L(+)-lactic acid isomer (figure 1).

Overall, the LAB group can utilize a wide range of carbohydrates, including the hexoses (glucose, fructose, mannose and galactose), as well as other pentoses, polyols and oligosaccharides. This capability is dependent on the species and strains involved, as well as the pH of the medium. Moreover, since malic acid cannot be used by wine LAB as a sole carbohydrate source (see below), the availability and utilization of fermentable carbohydrates in wine by LAB is essential to enable the onset of bacterial growth and the occurrence of MLF. Furthermore, recent studies have clearly demonstrated that grape-derived phenolic glycosides also significantly stimulate the growth of *O. oeni* in a synthetic wine medium.

MALOLACTIC CONVERSION – THE DEACIDIFICATION REACTION

Overall, three main pathways have been proposed for the degradation of L-malic acid to L-lactic acid by LAB during MLF. The first involves the activity of three separate enzymes – malate dehydrogenase, oxaloacetate decarboxylase and L-lactate dehydrogenase – and proceeds via the intermediates, oxaloacetic acid and pyruvic acid. A second mechanism proceeds via pyruvic acid and utilizes a combination of malic enzyme and lactate dehydrogenase. It was not until the 1970s that the enzymatic basis for this reaction was more fully elucidated in wine malolactic bacteria, specifically *Leuconostoc oenos* (*O. oeni*) ML34, by Kunkee 1975 and Morenzoni 1974. This work revealed that a single enzyme, commonly known as the "malolactic enzyme," exhibits two separate enzyme activities that act simultaneously on L-malic acid. The predominant "malolactic activity" of this enzyme (malate: NAD+ carboxylyase) catalyzes the direct conversion (decarboxylation) of the dicarboxylic acid L-malic acid to the monocarboxylic acid L-lactic acid, and requires NAD and Mn+2 as co-factors.

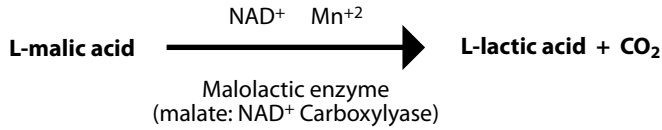

Figure 1. Malolactic fermentation

The malolactic enzyme from *L. oenos* (*O. oeni*) has a molecular mass of 138,000 and consists of two identical subunits, each with a molecular mass of 65,500 (Kunkee 1991).

ENERGETICS AND BIOLOGICAL ROLE

There has been considerable investigation in recent decades concerning the seemingly obscure benefit of malolactic conversion to the bacterial cell. The initiation of MLF in wine usually occurs after LAB have grown beyond a viable cell population of approximately 10^6 CFU/mL. Although providing deacidification and an accompanying increase in pH of up to approximately 0.2 pH units, the malolactic conversion itself appears energetically unfavourable to LAB. It yields little free energy (DG = -8.3 kJ/mole), proceeds without the formation of free intermediates and does not yield biologically available energy in the form of ATP. Further, although NAD is an essential co-factor, it does not serve an oxidation/reduction function as there is no net change in redox state (Pilone et al. 1976, Renault et al. 1988, Kunkee 1991, Henick-Kling 1993, and Boulton et al. 1998). In overall terms, MLF is not a true fermentation. In addition to supplying little energy for cell growth, it also does not supply a source of carbon for the biosynthetic reactions that are essential for cellular development. Nevertheless, the presence and utilization of malic acid appreciably stimulate the initial growth rate of malolactic bacteria, yet the resulting increase in pH that is associated with MLF does not fully account for this stimulatory effect (Pilone et al. 1976, Renault et al. 1988, and Boulton et al. 1998).

Although the conversion of L-malic acid to L-lactic acid by the malolactic enzyme is energetically unfavourable, the MLF has, in fact, been shown to provide energy in the form of ATP to the bacterial cell. This is accomplished by a chemiosmotic mechanism that generates a proton motive force (Dp) across the cell membrane. In this model, the MLF proceeds in three stages. In the first step, entry of L-malic acid into the bacterial cell is facilitated by a specific transport enzyme. In the second step, L-malic acid is decarboxylated within the cell by the malolactic enzyme, yielding L-lactic acid and CO_2, which then increases the intracellular pH. In the final stage, L-lactic acid and CO_2 are expelled from the cell. For every molecule of lactic acid that leaves the cell, one proton is also translocated outside of the cell. This establishes a proton gradient across the cell membrane between the cytoplasm and the surrounding

medium. This gradient, combined with a specific ATPase in the cell membrane, facilitates the generation of energy available for transport processes in the form of ATP. The synthesis of one ATP requires the entry of three protons through the membrane-bound ATPase (Cox and Henick-Kling 1989 and 1990, Henick-Kling 1993 and 1995, and Versari et al. 1999).

Malic and citric acids do not serve as the sole energy sources for the growth of LAB (Liu et al. 1995). Consequently, malolactic bacteria require sugars as a carbon source. However, under conditions of limiting sugar availability or of low pH, which inhibit sugar metabolism, energy (ATP) generated from MLF is beneficial to cell growth (Henick-Kling 1993). Another, but minor (<1%), activity of the malolactic enzyme has also been suggested to stimulate the metabolic activity and initial growth rates of wine LAB. This secondary malolactic enzyme activity (Morenzoni 1974) catalyzes the following reaction.

Figure 2. Secondary malolactic enzyme reaction

The very small amounts of pyruvic acid and $NADH_2$ generated by this secondary malolactic activity are considered to stimulate the initial stages of glucose metabolism and initial growth rates through the provision of hydrogen acceptors (Kunkee 1991, and Boulton et al. 1998).

In addition to the role of malolactic bacteria in conducting MLF, certain yeasts, including *Schizosaccharomyces pombe*, are also capable of catabolizing malic acid. However, this metabolism is not a true MLF since malic acid is metabolized to ethanol (Mayer and Temperli 1963, Bidan et al. 1974). Despite its potential for wine deacidification, drawbacks to using maloethanolic fermentation in yeast by species of *Schizosaccharomyces* include the formation of undesirable flavour compounds, such as hydrogen sulphide (Bidan et al. 1974, Gallander 1977, Rankine 1966, Snow and Gallander 1979, and Davis et al. 1985).

THE EFFECTS OF MALOLACTIC FERMENTATION ON OVERALL WINE COMPOSITION AND QUALITY

• Acidity reduction

For each molecule of L-malic acid catabolized to the weaker L-lactic acid through MLF, there is a stoichiometric loss of a carboxyl group and corresponding reduction in wine acidity. In addition to the dependency of such effects on the initial concentration of malic acid, the

actual changes in wine acidity and pH attributable to MLF depend on other factors, including the buffering capacity of the wine as well as the initial pH (Boulton et al. 1998). In general, the overall decrease in wine acidity resulting from MLF can vary from 1 to 3 g/L, and pH may rise by 0.1 to 0.3 pH units (Davis et al. 1985). Wines produced from grapes cultivated in cool climate viticultural areas contain a naturally high level of malic acid of up to approximately 8 g/L, and are considered to benefit from such an acid reduction. On the other hand, wines produced from grapes grown in warm-to-hot regions have lower total acidity (4.5 to 5.5 g/L), and a further reduction in acidity from MLF can have a negative impact on wine quality, causing a flat taste and a greater predisposition to bacterial spoilage (Rankine 1972). Nevertheless, MLF can be desired in such wines to confer a degree of biological stability and/or to impart flavour complexity, necessitating the use of acidulants to adjust wine acidity and pH to acceptable levels after MLF. The increase in wine pH accompanying MLF can also influence wine colour.

• Flavour changes

Although there has been conjecture over the contribution of MLF to the sensory properties of wine (Davis et al. 1985), more recent research has provided greater insight into specific sensory changes associated with the growth and metabolic activity of malolactic bacteria in wine. It is clear that different strains of malolactic bacteria may increase or decrease the intensity of certain wine aroma and flavour attributes, and such changes are strain dependent (Bartowsky and Henschke 1995, and Bartowsky et al. 2011). In addition to deacidification, the flavour attributes imparted by MLF can be described as buttery, lactic, nutty, yeasty, oaky, sweaty and earthy. MLF may also impact fruity and vegetative aromas, as well as the mouthfeel of wine (Henick-Kling 1993, Henick-Kling et al. 1993, and Laurent et al. 1994). Mechanisms by which malolactic bacteria can influence wine flavour may include (i) removal of existing flavour compounds by metabolism and adsorption to the cell wall, (ii) production of new bacterial-derived flavour compounds from the metabolism of sugars, amino acids and other substrates, and (iii) metabolism and modification of grape- and yeast-derived secondary metabolites to end products having greater or lesser sensory impact (Bartowsky and Henschke 1995). Importantly, the net impact of MLF on wine sensory properties will depend on factors such as bacterial strain characteristics, varietal aroma intensity of the wine and vinification techniques employed (Henick-Kling 1995). The following sections outline some of the important flavour compounds and sensory effects associated with the metabolism of malolactic bacteria in wine.

PRODUCTION OF DIACETYL – THE METABOLISM OF CITRIC ACID

LAB are well known for their ability to produce diacetyl (2,3-butanedione), an intensely aromatic diketone that is characterized by a buttery, nutty aroma. Although small quantities of diacetyl (0.2 to 0.3 mg/L) can be produced by the alcoholic fermentation of yeast, subsequent increases in diacetyl content are typically associated with the growth of LAB and MLF (Laurent et al. 1994, Martineau et al. 1995, Davis et al. 1985, and Bartowsky and Henschke 2004). The aroma threshold of diacetyl in wine is low (0.2 to 2.3 mg/L) and is dependent on wine type (Martineau et al. 1995). Depending on the style and type of wine, the production of low amounts of diacetyl (1 to 4 mg/L) contribute a buttery sensory character and are considered desirable. However, the formation of concentrations in excess of 5 to 7 mg/L can be detrimental to wine quality and may cause spoilage (Rankine et al. 1969, and Davis et al. 1985).

Diacetyl is produced by *O. oeni* as an intermediate in the metabolism of citric acid. In this pathway, the intermediate, pyruvic acid, is reductively decarboxylated to diacetyl via a-acetolactate. Since diacetyl is chemically unstable, it can be further reduced by active cells of *O. oeni* or by yeast to less flavour-active end products, the keto-alcohol acetoin and the diol, 2,3 butanediol (Ramos et al. 1995, Bartowsky and Henschke 2004, and Bauer and Dicks 2004). In addition to citric acid, the metabolism of diacetyl by LAB is closely associated with the metabolism of sugars and malic acid as shown in figure 3… *(Complete text available in printed version of the book.)*

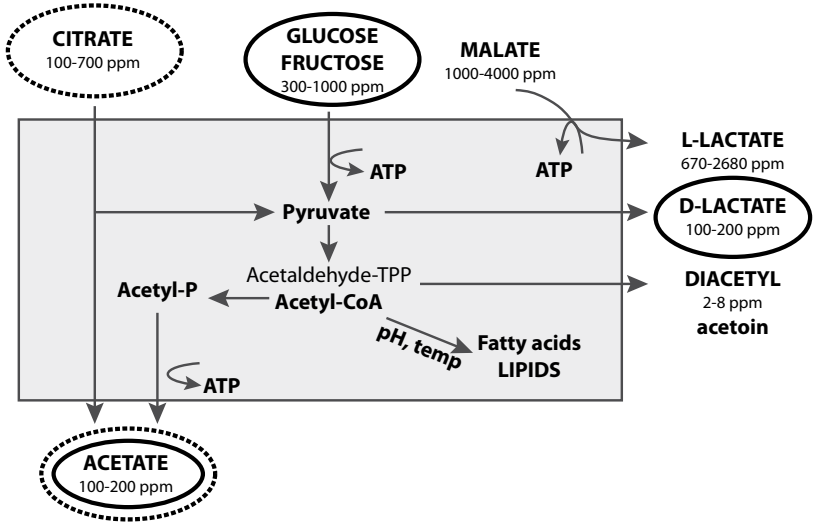

Figure 3. Metabolism of heterofermentative lactic acid bacteria

(Source: Krieger 2005)

WINE SENSORY COMPONENTS AND MALOLACTIC FERMENTATION

RICH MORENZONI, PH.D.

Malolactic fermentation (MLF) is the well-documented decarboxylation of L-malic acid to L-lactic acid and CO_2 by wine lactic bacteria (LAB). It is a biological process conducted by a living organism, which means that lactic acid and CO_2 are not the sole end products of the reaction. The causative bacteria cannot grow on L-malic acid as the only carbon source. They require carbohydrates and nitrogen, as do all living organisms. These bacteria derive the majority of energy for growth by metabolizing the various sugars present in grape juice and/or wine. The decarboxylation of malic acid supplies only a very small amount of the energy required to sustain microbial growth. As a corollary to the bacterial metabolism of carbohydrates and amino acids, a variety of metabolic end products can be produced. Some of the compounds produced have pleasing organoleptic and sensory properties, while some do not. Recently, Sumby et al. (2013) have demonstrated the presence of esterases in wine MLB and have clearly shown they can modify the ester profile during the course of MLF in wine. The success and usefulness of selected MLB strains rest in their ability to produce desired products with minimal or no production of undesirable products. Malolactic bacteria possess the ability to affect wines in a positive way, both texturally and sensorially.

The most common wine LAB currently in use is *Oenococcus oeni.* Many strains of this organism are available, and they can affect the organoleptic profile as well as the body and texture of wine. Some common terms used to describe the positive effects of MLF on wine flavour are nutty, toasty, buttery, fruity and spicy. These descriptors can be attributed to the production of flavour-active compounds, or to the modification of existing flavour components. In addition, the body of the wine can be affected, making it softer and richer.

Historically, MLF was not well controlled in wine production, and its effect was often less than desirable. If MLF proceeded to completion without imparting flavours uncomplimentary to the organoleptic profile of the wine, MLF was considered a success. Current wine production techniques are much more sophisticated, as are the strains of wine lactic acid bacteria available to the winemaking community. The impact on wine flavour from MLF can now be attributed to the way the wine LAB either modify the existing flavour compound precursors, which occur naturally in grape juice and wine, or to the production of specific flavour compounds as the bacteria grow in the product. Indeed, winemakers are now capable of "sculpting" wines by making use of specific bacteria and their ability to modify the aroma, texture, mouthfeel and organoleptic profile of the product.

The impact of MLF on the body of wines can be attributed to the decarboxylation reaction itself, as well as to the formation of metabolic by-products. When MLF occurs, the dicarboxylic acid, L-malic acid, is converted to the monocarboxylic acid, L-lactic acid. There will be a concomitant increase in pH because of the conversion of one of the acidic carboxyl groups of malic acid into CO_2. On average, after MLF is complete, the pH will increase by approximately 0.2 units. This tends to make the wine less acidic, softer and texturally more pleasing in the mouth.

As a result of the growth of MLB in wine, it is often felt that a degree of biological stability toward bacterial growth is imparted. Winemakers generally feel that once their wine has successfully passed through MLF, clarification and stabilization of the product can be performed. The use of selected MLB strains in the winemaking process has played a large role in achieving early biological stability for the product. These strains of wine LAB have been well researched and exhaustively catalogued. They confer upon the winemaking process the same degree of reliability and predictability as the known strains of active dry wine yeast, which are routinely used to conduct the alcoholic fermentation (AF).

In the past, MLF has been used mainly in the production of red wines, and sometimes in the production of white wines. In white wine production, MLF is usually used to lower the acidity of wines produced in cool climates or to confer a degree of organoleptic complexity to such lesser-flavoured white varieties as Chardonnay. Recent research has begun to explore the effects of MLF on white wines from varied viticultural regions. The malolactic organisms are subject to the same growth necessities in white wine as in red wine, but when they interact with the components of the white wine matrix, the products they form can vary. This is very interesting and helpful, because it can aid the winemaking community in its quest to craft a product with a unique, pleasing and characteristic organoleptic profile.

In addition to the use of wine MLB strains to conduct and complete MLF, the possibility arises that the many available strains can achieve varying sensory profiles in different products. Wine LAB can be added during the winemaking process in two ways: 1) with the wine LAB inoculation of the must 24 to 48 hours after the addition of fermentative yeast, or 2) with wine LAB inoculation at or near completion of AF. Both techniques can impart different flavour profiles and can avoid the production of some undesirable traits; both these techniques will be discussed later. Similarly, the use of a specific wine LAB strain with a specific type of grape may increase or decrease the levels of desirable or undesirable compounds, flavours and characteristics in that product. In this chapter we will look at work that has concentrated on techniques to improve and/or control the flavour impact on wines.

THE EFFECTS OF PH AND ETHANOL

Wine LAB have long been known to produce different compounds depending on the pH of the wine in which they are growing. Bartowsky et al. (2011), when using a Cabernet Sauvignon wine produced from four distinct Australian viticultural areas, demonstrated a different and distinct metabolite profile when the MLF was conducted at low vs. high pH (figure 1). They used three strains of *O. oeni* – VP41, Beta and Elios 1 – and found that generally all three produced higher amounts of the C4-C10 ethyl esters at pH 3.3 than at pH 3.7. This is a significant observation because that group of volatile esters imparts certain fruity characters to the wine. The authors further state that total "red fruit" esters were higher at the lower pH. At the higher pH, these esters were either absent or present in much lower amounts. The flavour descriptors of the volatile esters produced were described as fruity and raspberry-like and were a positive addition to the overall flavour of the Cabernet Sauvignon wine. Figure 1 shows that wines receiving an inoculation of any of the MLB strains not only generally exhibited higher ester levels than did control wines without MLF, but the strains used exhibited large differences in the amounts produced in relation to the pH of the wine. Ester production is a biological phenomenon, and MLB generally have been known to slightly increase the levels of ethyl esters of acetic and lactic acids. At normal levels, these compounds are not necessarily flavour-active, but the ones produced by the *O. oeni* strains VP41, Beta and Elios 1 contribute to the fruity character of the wine, and VP41 and Beta, especially, consistently produce red wines with increased sensory characters related to the red fruit profile. These observations are significant as historically MLF was said to destroy the fruity character of wine. It is now known that by choosing the appropriate bacterial strain to use in the appropriate juice/wine, fruitiness is no longer lost but can actually be accentuated.

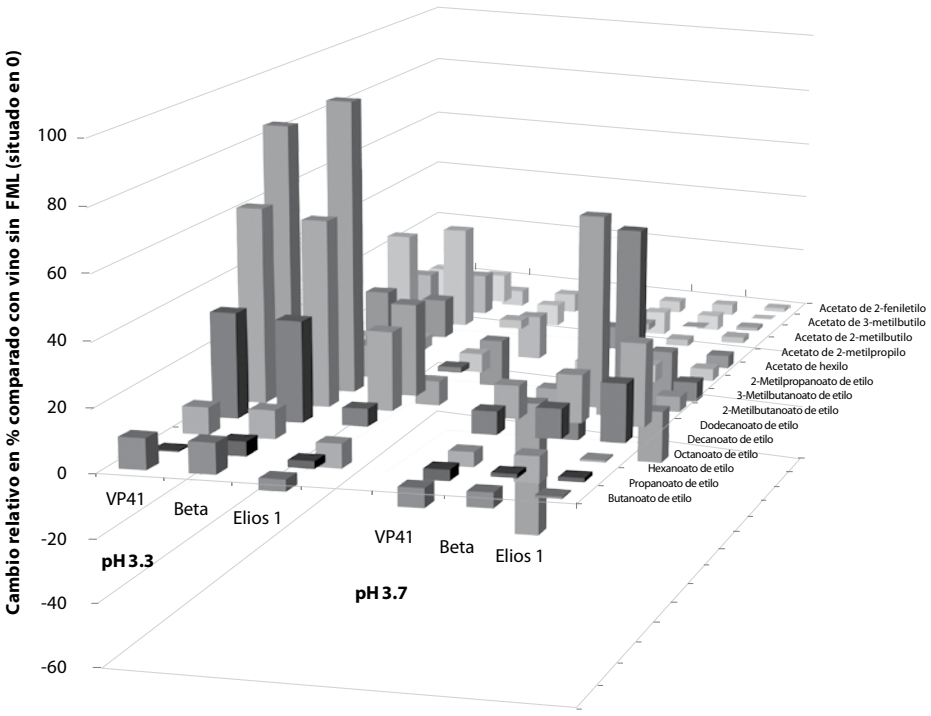

Figure 1. Volatile flavour compounds of Cabernet Sauvignon wine as influenced by malolactic bacterial strains (Source: Bartowsky et al. 2011)

Knoll et al. (2011) examined the influence of pH and ethanol on the aroma compounds associated with MLF in white wine varieties. They reported only small changes in the higher alcohol profile, but stated that rose and floral notes (2-phenylethanol) were increased at lower ethanol levels, while the concentration of the ethyl esters of hexanoic, octanoic and decanoic acids were increased at higher alcohol levels. They found that pH and ethanol were the primary components to influence the volatile component profile of Riesling wine after it completed MLF. The principal component analysis (PCA) plot of their findings is shown in figure 2. The profiles associated with pH 3.2 are clearly different from profiles associated with pH 3.6. Ethyl esters of succinic acid correlate with lower pH, and acetic acid 3-methylbutylesters correlate with higher pH. Generally, the longer chained esters correlate with higher alcohol contents, while hexanol correlates with lower alcohol levels… (*Complete text available in printed version of the book.)*

MICROBIOLOGY OF MALOLACTIC FERMENTATION

NICHOLA HALL, PH.D., AND SIBYLLE KRIEGER-WEBER, PH.D.

In an oenological environment, the micro-organisms involved with the utilization of L-malic acid are primarily bacteria, with yeast playing a minor role.

Bacteria convert the L-malic acid from grapes into L-lactic acid and carbon dioxide, whereas certain yeasts can convert the L-malic acid to ethanol. Although many genera of bacteria can produce lactic acid (table 1) as either a primary or a secondary end product of sugar fermentation, the term "lactic acid bacteria" (LAB) is conventionally reserved for members of the *Lactobacteriaceae* family, which include the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus* (Davis et al. 1985, and du Toit and Pretorius 2000), among others. These organisms are classified as Gram-positive, non-spore-forming bacteria, which are either cocci, coccobacilli or bacilli that have a G:C ratio of less than 53%. The members of this bacteria group are generally non-respiratory and lack a heme-containing catalase. Collectively termed LAB, they can be isolated from the grapes, leaves and soil, as well as from the surfaces of winery equipment. They generally grow best under conditions of limited oxygen, and require carbohydrates and certain preformed amino acids, peptides, minerals and vitamins in order to proliferate (Henick-Kling 1988, Wibowo et al. 1985, and Terrade and Mira de Orduña. 2009).

Recent advances in molecular tools have permitted the fast and sensitive characterization of the majority of wine LAB, whether indigenous isolates or commercially available cultures. The current taxonomic classifications have been based on polymerase chain reaction (PCR) techniques. However, the intra-species diversity of LAB and strain typing are studied by enzymatic restriction coupled with restriction nuclease analysis by pulse-field gel electrophoresis (REA-PFGE), and more recently by multilocus sequence typing (MLST). These techniques suggest that *Oenococcus oeni* could be divided into two sub-species (Bilhère et al. 2008, and Claisse et al. 2012).

Typically, the indigenous LAB isolated from grape musts are present at approximately $1x10^4$ CFU/mL. The majority of these bacteria are not tolerant toward the environmental conditions associated with winemaking, but some are capable of withstanding the challenges of alcoholic fermentation (AF) (Lonvaud-Funel et al. 1991). It appears the pH of the environment is the key driver of LAB population dynamics. At a pH below 3.5, the dominant LAB is *O. oeni,* while wines exhibiting a pH greater than 3.5 are capable of supporting a much broader range of species. This group of organisms is comprised of *Lactobacillus brevis* (Vaughn 1955, Du Plessis and van Zyl 1963, Pilone et al. 1966, Chalfan et al. 1977, Sharpe 1981, Dicks and van Vuuren 1988, and Edwards et al. 1993), *L. buchnerii* (Vaughn 1955, Du Plessis and van Zyl 1963, Pilone et al. 1966, and Sharpe 1981), *L. casei*, *L. curvatus*, *L. delbrueckii*, *L. fermentum* (Vaughn 1955), *L. fructivorans* (Amerine and Kunkee 1968, and Edwards et al. 1993), *L. hilgardii* (Vaughn 1955, Du Plessis and van Zyl 1963, Dicks and van Vuuren 1988, and Edwards et al. 1993), *L. jensenii*, *L. kunkeei* (Edwards et al. 1998), *L. nagelii* (Edwards et al. 2000), *L. plantarum* (Wibowo et al. 1985, Edwards et al. 1993, and Benduce et al. 2004), *L. sakei* (Kandler and Weiss 1986), *L. desidiosus*, *Pediococcus parvulus*, *P. damnosus* (formerly *P. cerevisiae*), *P. pentosaceus* (Wibowo et al. 1985, Edwards and Jensen 1992, Fugelsang 1997, Gindreau et al. 2001, and Benduce et al. 2004), *Leuconostoc mesenteroides* (Lafon-Lafourcade et al. 1983, Lonvaud-Funel 1999, and Ribéreau-Gayon et al. 2000) and *Leuconostoc gracile* (Ribéreau-Gayon et al. 1975), as well as *O. oeni* (Lafon-Lafourcade et al. 1983, Wibowo et al. 1985, Dicks et al. 1995, Fugelsang 1997, and Ribéreau-Gayon et al. 2000).

Regardless of the type of LAB present, the primary role of these organisms in wine production is to convert L-malic acid to L-lactic acid and carbon dioxide, thus resulting in a change of wine acidity. This biological conversion of L-malic acid to L-lactic acid is termed malolactic fermentation (MLF) (Davis et al. 1985, Liu 2002, and Rankine et al. 1970).

MLF not only results in a biological deacidification, it can exert a significant impact on the organoleptic qualities of wine. These sensory effects can be positive, negative or neutral depending on the bacterial strain present and, more specifically, the strain of LAB employed to conduct the MLF. For more detailed information, see the review by Liu (2002). LAB strains that negatively influence the final product may cause a range of undesirable changes to wine sensory properties, such as masking varietal fruit characters, altering wine colour and producing undesirable metabolites, e.g., biogenic amines (Davis et al. 1985).

Species of *Lactobacillus* or *Pediococcus* (figure 1) may conduct MLF, especially in wine with a pH of 3.5 or higher, but it was thought that they produced wines of unacceptable quality (Krieger et al. 1990). Although ethanol tolerant, these genera of bacteria are poorly tolerant to low pH conditions and can produce undesirable flavours. Currently, we are reviewing spe-

cies of *Lactobacillus*, especially *Lactobacillus plantarum* (Murphy et al. 1985, Mtshali et al. 2009, Lerm et al. 2011, and Fumi et al. 2010), as potentially viable malolactic bacteria (MLB) candidates. These species are interesting options, especially when we consider their sugar utilization pathways and resulting metabolites. Some species of *Lactobacillus* (e.g., *L. casei* and *L. plantarum*), as well as *Pediococcus,* are classed as homofermentative or facultative heterofermentative and produce lactic acid as the end product of hexose metabolism (table 1). The obligatory heterofermentative LAB, which include some species of *Lactobacillus* (e.g., *L. brevis*, *L. hilgardii* and *L. kunkeei*) and *O. oeni*, produce carbon dioxide, acetic acid and ethanol in addition to D-, L-, and DL-lactic acid as end products of carbohydrate metabolism (table 1).

Table 1. Carbohydrate metabolism of lactic acid bacteria

Group	Glucose fermentation	Lactic acid isomer	Species
Lactobacilli (rod-shaped cells)	Facultative heterofermentative Group 2	L	*Lactobacillus casei*
		DL	*Lactobacillus plantarum*
	Obligatory heterofermentative Group 3	DL	*Lactobacillus brevis*
		DL	*Lactobacillus hilgardii*
		L	*Lactobacillus kunkeei*
Cocci (round cells)	Obligatory homo-fermentative	DL	*Pediococcus damnosus*
		DL	*Pediococcus pentosaceus*
	Obligatory hetero-fermentative	D	*Leuconostoc oenos (Oenococcus oeni)*
		D	*Leuconostoc mesenteroides* subspecies *mesenteroides*

At this time, strains of *Pediococcus* and *Lactobacillus* are not used for commercial wine production in the American market. There is a perceived lack of suitable organisms, but this may change as *Lactobacillus plantarum* MLF starter cultures have recently been introduced to the international wine industry because of their interesting enzymatic properties and their homofermentative behaviour with regard to hexose sugars.

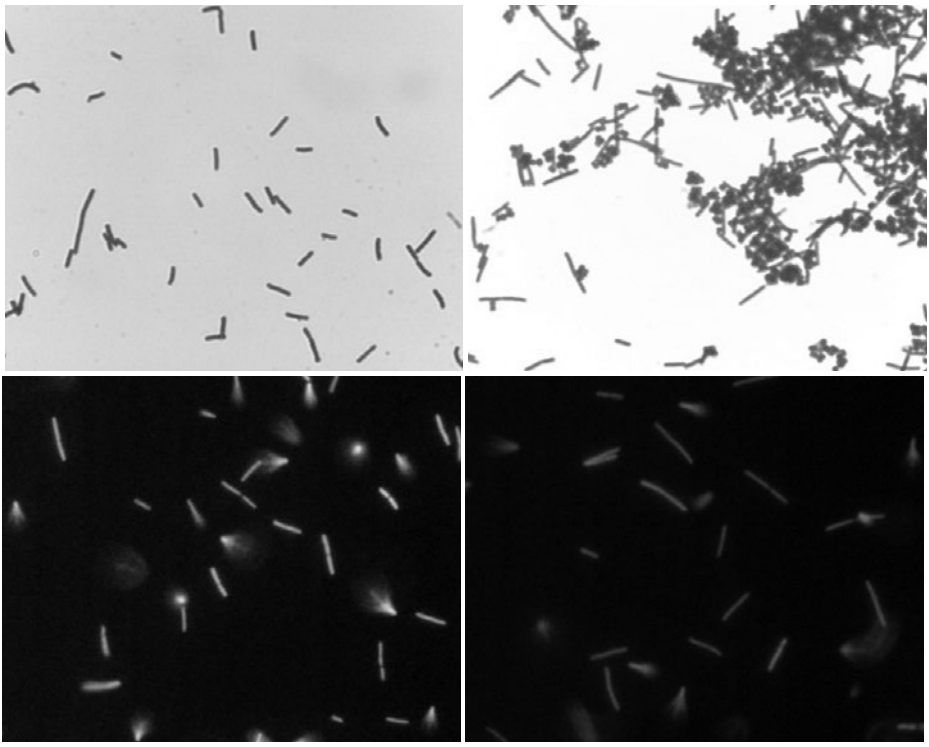

Figure 1. Gram-stained *Lactobacillus brevis* (top left), a mixture of Gram-stained *Pediococcus damnosus* and *Lactobacillus buchnerii* (top right), fluorescent-stained live (bottom left) and dead (bottom right) *Lactobacillus brevis* cells. Courtesy of ETS Laboratories.

Yeast species that are recognized for their ability to metabolize L-malic acid are able to decompose L-malic acid through a process known as maloethanolic fermentation (MEF). MEF is carried out mostly by such yeasts as *Schizosaccharomyces pombe,* which may decompose up to 100% of L-malic acid, and strains of *Saccharomyces cerevisiae,* which may be able to consume up to 40% of the L-malic acid. These yeasts convert L-malate into pyruvate by means of an intracellular malic enzyme (Volschenk et al. 2003), which is then converted to ethanol rather than to L-lactic acid. The MEF is distinct from the bacteria-catalyzed MLF, although the end result is comparable in terms of the degradation of L-malic acid. It is estimated that 2.3 g/L of L-malic acid is converted into 0.1 g/L of ethanol. The production of L-lactic acid by yeast is greatly influenced by culture conditions. It is favoured by high sugar concentrations (20% to 30%), pH values of about 5, limiting levels of nitrogen (100 to 250 mg N/L), and the presence of CO_2 (Radler 1993). The MEF does not have a significant influence on the final ethanol concentration… *(Complete text available in printed version of the book.)*

ORGANOLEPTIC DEFECTS CAUSED BY UNCONTROLLED MALOLACTIC FERMENTATION

JOSÉ MARÍA HERAS

The evolution of winemaking practices has not only led to improvements in wine quality, it has made it possible to emphasize the grapes' organoleptic properties. Biological control of the winemaking process optimizes the alcoholic fermentation (AF), and the judicious choice and use of winemaking biologicals may also influence wine style. In order to maximize the expression of grape-derived wine parameters, controlling the winemaking process is paramount. This begins with carefully choosing the yeast to conduct the AF and selecting the appropriate wine lactic acid bacteria (LAB) to conduct the malolactic fermentation (MLF), and is followed by sound technological winemaking practices.

Modern winemaking places increasing importance on maximizing the positive sensory attributes of the product, while minimizing or eliminating negative ones. Indeed, sensory components that affect the product negatively may have a greater impact than those that affect the product positively. Beneficial properties, such as fruitiness and pleasant tannins, may be masked by obvious, identifiable defects.

MLF is an important step in the winemaking process, not only reducing acidity by converting L-malic acid to L-lactic acid, but also influencing the sensory profile of the wine with the compounds produced by the wine LAB as a consequence of their growth and metabolism. In moderate concentrations, compounds such as ethyl lactate and diacetyl can exert a positive influence. Wine LAB can reduce vegetal notes, astringency and bitterness while contributing to roundness and positive tannin expression. Recent findings have shown certain strains of

selected wine LAB are capable of increasing fruit ester concentrations in red wine (Costello et al. 2012) and in white wine (Knoll et al. 2011). Using selected wine LAB to induce MLF increases the winemaker's control over these sensory expressions.

Wines that have completed a controlled MLF are described with such positive sensory descriptors as buttery, nutty, yeasty, honey, vanilla, spicy, earthy, toasty and fruity. Texturally, these wines are described as having more body and roundness, greater length on the palate and silky tannins. Conversely, wines that have undergone uncontrolled MLF, especially at pH levels greater than 3.5, are described with such negative descriptors as intense lactic aroma, acid yogurt, rancid, sweaty, acetic, intense bitterness and animal notes (table 1).

Table 1. Negative descriptors of uncontrolled malolactic fermentation

Problem	Condition for occurrence	Implicated organisms	Compound modified	Compound created	Resulting effect on wine
Ropiness (oily wines)	Usually just white wines	*Streptococcus mucilaginosus*, *Pediococcus parvulus*, *P. damnosus*, *Leuconostoc mesenteroides*	Glucose	Glucose-containing mucilaginous polysaccharides, glucans	Increased viscosity, filtration problems
Volatile phenol production	High pH red wines	Some *Pediococcus* and *Lactobacillus* (mainly from *Brettanomyces* and *Dekkera* yeasts)	Hydroxycinnamic acids	4-VP, 4-VG, 4-EP, 4-EG, 4-EC	Includes horse sweat, horse stable, leather, asphalt, mould, medicine, smoke
Biogenic amine development	High pH wines	Lactic acid bacteria	Certain amino acids	Histamine, tyramine	Human health concerns
				Putrecine, cadaverine	Putrefaction, meaty, vinegary, dirty aromas

Problem	Condition for occurrence	Implicated organisms	Compound modified	Compound created	Resulting effect on wine
Mousiness	High pH, oxidative conditions	Hetero-fermentative *Lactobacillus*, *O. oeni* (possibly also *Brettanomyces* and *Dekkera* yeasts)	Amino acids (lysine, ornithine), sugars	Pyridines	Mousiness
Masking of varietal aromas	Red and white wines	Certain malolactic strains	Organic acids, sugars, amino acids	Diacetyl, ethyl lactate, ethyl acetate	At lower levels masks fruit character, at higher levels nutty, yeasty, lactic and wet fur aromas
Glycerine decomposition	Reds or whites with low alcohol, high pH wines (especially press wines and wines with prolonged lees aging)	*L. casei*, *L. fructivorans*, *L. hilgardii*	Glycerine	Acrolein	Bitterness
Tartaric acid decomposition	Reds or whites with pH >3.5, low total acidity	*Pediococcus*, *Lactobacillus*	Tartaric acid	Lactic acid, acetic acid, CO_2	Acidity decrease, volatile acidity, colour loss, cloudiness
				Acetamide (rare)	Mouse urine aroma (rare)
Fermentation of sugars in wines with stuck alcoholic fermentation	Red or whites with sugar available for LAB metabolism, such as found in stuck alcoholic fermentations	Most LAB	Fermentable sugars	Lactic and acetic acids	Increase total acidity, VA, loss of complexity and balance, cloudiness
			Fructose	Mannitol	Bittersweet taste

Problem	Condition for occurrence	Implicated organisms	Compound modified	Compound created	Resulting effect on wine
Metabolism of non-fermentable sugars in dry wines – AF completed	Dry wines, especially high pH reds	Most LAB	Arabinose, xylose, glucose, fructose	Acetic acid	VA

ORGANOLEPTIC DEFECTS ASSOCIATED WITH MALOLACTIC FERMENTATION

Ropiness (oily wine)

Ropy wine is characterized by an atypically high viscosity and an oily or slimy appearance. Ropiness is caused by production of the polysaccharides glucan or dextran from glucose. Synthesis of polysaccharides by LAB is a widespread characteristic, with dextran production by *Leuconostoc mesenteroides* being the best-known example. Van Vuuren and Dicks (1993) reported that some *Oenococcus oeni* strains have a "slimy" layer around their cells. *Pediococcus damnosu*s (Mayer 1974), as well as *Pediococcus* sp., *Streptococcus mucilaginous* and *Lactobacillus pasturianus* also have this ability. In wine, 50 to 100 mg/L of glucose may be sufficient to allow the formation of polysaccharides. Ropiness may occur in tanks, barrels or the bottled product. It usually occurs in white wines and is very rare in reds because the causative organisms do not grow well in the presence of tannin.

Volatile Phenols

The negative impact of volatile phenols produced by *Brettanomyces bruxellensis* growth in wines is a major threat to wine quality. This yeast will grow in wine during maceration or while waiting to undergo MLF, even under conditions of high alcohol, high SO_2 and limited nutrient availability, and will produce the undesired compounds 4-ethylphenol, 4-ethylgaiacol and 4-ethylcatechol. These volatile phenols are characterized by animal-like odours described as horse and barnyard, pharmaceutical odours characterized as band-aid and medicinal, as well as aromas associated with ink.

It is interesting to note that *Brettanomyces* is not the only micro-organism capable of producing volatile phenols. *L. brevis, L. collinoides, L. plantarum, L. mali, L. sake, P. damnosus and P. pentosaceus* are also able to produce volatile phenols (Coutu et al. 2006). Similar results were observed with some strains of *L. plantarum* by Fras (2014). The pathway of the production of volatile phenols by *L. plantarum* is shown in figure *1. (Complete text available in printed version of the book.)*

NUTRITION OF MALOLACTIC BACTERIA

MAGALI DÉLÉRIS-BOU AND SIBYLLE KRIEGER-WEBER, PH.D.

Wine lactic acid bacteria (LAB) are known to have complex nutritional needs for carbon, nitrogen, vitamins and minerals. When musts or wines are deficient in one or more of these components, a major impact on malolactic fermentation (MLF) may be observed. In this chapter, the known nutritional requirements of wine LAB will be discussed at length, as well as the use of tools to facilitate the onset and progress of MLF.

NUTRIENTS

Carbohydrates

In wine, sugars are the primary source of energy for LAB and play an essential role in the growth of LAB. The main sugars found in must and wine are the hexoses – glucose and fructose. LAB are capable of utilizing both, although *Oenococcus oeni* prefers fructose. However, the cometabolism of glucose and fructose is advantageous in terms of energy production.

At the end of alcoholic fermentation (AF), the glucose and fructose concentrations are low. Nevertheless, both hexoses are present in sufficient levels to meet the carbon requirements of the selected wine LAB that were inoculated into the wine. Most wine LAB are capable of utilizing the monosaccharides arabinose, mannose, galactose and xylose, etc., as well as polysaccharides and glycosylated compounds. Glycosides represent a latent pool of aroma compounds, where the aglycone is conjugated to glucose and a second sugar molecule via glycosidic bonds, which require either heat or enzymatic activity to release the active aroma compound. *O. oeni* and other wine LAB species possess this glycosidase activity (Guilloux-Benatier et al. 1993, Guilloux-Benatier et al. 2000, Grimaldi et al. 2000, MacMahon et al. 1999, and Mansfield et al. 2002). Wine LAB glycosidase action will cleave this bond, releasing aroma precursors, as well as glucose, which may be used as a carbon source for further bacterial growth.

Organic Acids

L-malic acid

The concentration of L-malic acid in grape must depends on grape maturity and varies from 0.7 to 8.6 g/L (Cabanis and Cabanis 1998). The main reaction of MLF is the decarboxylation of L-malic acid to L-lactic acid. This decarboxylation, in addition to increasing wine pH and decreasing acidity, allows *O. oeni* to derive a small but measurable amount of energy in the form of adenosine triphosphate (ATP) and to maintain an intracellular pH favourable for enzyme activity and cell growth.

L-malic acid passes into the cell in its protonated form to be decarboxylated into L-lactic acid in the cell's cytoplasm. The decarboxylation allows the consumption of an intracellular proton and the expulsion of protons by lactate/ H+ symporters. A proton gradient, or "proton-motive force," allows the intracellular pH to increase to approximately 6.0, leading to ATP formation.

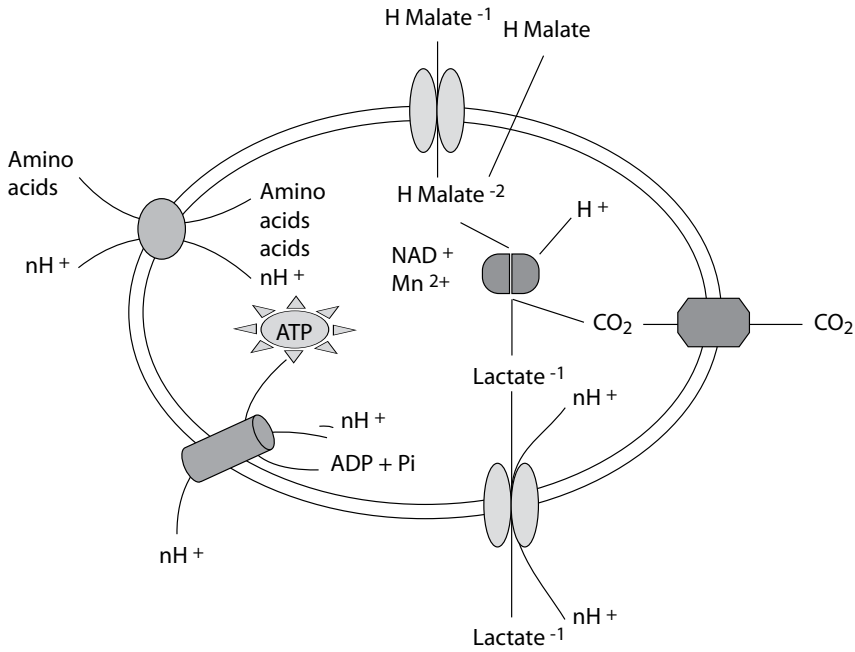

Figure 1. Proton motive force and ATP generation during malolactic fermentation

Figure 1 shows the consumption of intracellular protons during L-malic acid decarboxylation and the proton motive force generated by the malic acid metabolism. L-malic acid enters the bacterial cell through a permease mechanism and is enzymatically converted to L-lactic

acid. The resultant L-lactic acid is then conveyed out of the cell and into the medium by a permease system. The proton gradient generated by the conversion of L-malic acid to L-lactic acid leads to ATP formation through the action of an ATPase pump. The derived ATP may be involved in subsequent amino acid transport (Desroche 2005).

Citric acid

Citric acid is a component of must and wine, and is present in a quantity between 0.1 and 0.7 g/L. Not only is the degradation of citric acid by wine LAB a source of energy, it results in the formation of acetic acid, lipids, acetoin, butanediol and diacetyl. In *O. oeni*, the timing and speed of citrate decomposition is strain specific. While some strains that produce high levels of diacetyl begin to consume citric acid from the midpoint of the MLF, other strains do not start metabolizing citric acid until all the L-malic acid has been depleted (Krieger 2012).

Nitrogen – Amino Acids and Peptides

Free amino acids, as well as those arising from the hydrolysis of peptides, are the main sources of the nitrogen used by wine LAB. Contrary to yeast, the amino acids required for wine bacteria cannot be synthesized from inorganic nitrogen. They must either be supplied by the wine matrix or be synthesized by the metabolism of wine LAB from organic sources.

Amino acids

The amino acid requirements of wine LAB depend not only on the bacterial species but on the strain. The identification of essential amino acids for *O. oeni* growth has been the subject of numerous studies. Garvie (1967), Fourcassié et al. (1992), Remize et al. (2006), and Terrade and Mira de Orduña (2009) collectively have explored the needs of 22 strains of *O. oeni*. The technique used to determine the amino acid requirements consists of comparing the growth of the wine LAB in a complete medium containing all known required amino acids with the growth of the bacteria in a medium from which these amino acids are individually eliminated. If an amino acid is required, the amount of biomass formed will be less than that of the control. If, however, the amino acid is not required, there will be very little difference in the amount of biomass formed as compared to the control. The results are not always consistent, and the discrepancies may be explained by the varying methodologies used. A summary of the findings is shown in table 1.

Table 1. Amino acid requirements of 22 strains of *Oenococcus oeni*
Source: Alexandre et al. 2008, and Terrade and Mira de Orduña 2009

	Garvie 1967	Fourcassié et al. 1992	Remize et al. 2006	Terrade et al. 2009
Glutamic acid	Essential	Essential	Essential	Essential (1/2) Indifferent (1/2)
Arginine	Essential	Essential	Essential	Essential
Isoleucine	Essential	Essential	Essential (3/5) Necessary (2/5)	Essential
Tryptophan	Essential (7/9) Necessary (2/9)	Essential	Essential (4/5) Necessary (1/5)	Essential
Methionine	Essential (4/9) Necessary (5/9)	Essential	Essential	Essential
Valine	Essential	Essential (3/6) Necessary (3/6)	Essential (4/5) Necessary (1/5)	Essential
Cysteine	Essential	Essential (4/6) Indifferent (2/6)	Essential (1/5) Necessary (4/5)	Essential
Tyrosine	Essential	Essential (1/6) Indifferent (5/6)	Essential	Essential
Phenylalanine	Essential (7/9) Necessary (2/9)	Essential (1/6) Indifferent (5/6)	Essential	Essential
Histidine	Essential (6/9) Necessary (3/9)	Essential (1/6) Indifferent (5/6)	Essential (3/5) Necessary (2/5)	Essential
Serine	Essential (2/9) Necessary (5/9) Indifferent (2/9)		Essential	Essential (1/2) Necessary (1/2)
Lysine	Indifferent	Essential (1/6) Necessary (3/6) Indifferent (2/6)	Essential (1/5) Necessary (4/5)	
Aspartic acid	Necessary (3/9) Indifferent (6/9)	Necessary	Essential (1/5) Necessary (3/5) Indifferent (2/5)	
Leucine	Essential (1/9) Necessary (1/9) Indifferent (5/9)	Essential (3/6) Necessary (3/6)	Essential (4/5) Necessary (1/5)	Essential
Threonine	Essential (1/9) Necessary (1/9) Indifferent (7/9)	Indifferent	Essential (1/5) Necessary (2/5) Indifferent (3/5)	Essential
Glycine	Essential (2/9) Necessary (1/9) Indifferent (6/9)	Indifferent	Essential (1/5) Necessary (2/5) Indifferent (3/5)	Essential
Proline	Indifferent	Indifferent	Necessary (3/5) Indifferent (3/5)	Essential
Alanine	Indifferent	Indifferent	Necessary (1/5) Indifferent (4/5)	

(Complete text available in printed version of the book.)

ENVIRONMENTAL FACTORS AFFECTING MALOLACTIC FERMENTATION

SIBYLLE KRIEGER-WEBER, PH.D., ANTHONY SILVANO AND PIET LOUBSER

INTRODUCTION

Malolactic fermentation (MLF) is generally considered to be a simple bacterial breakdown of L-malic acid in red wines and some white wines, with the accompanying release of CO_2, the formation of L-lactic acid and a reduction in the total acid content. The simple conversion of L-malic acid to L-lactic acid could be an oversimplification of the process, as MLF involves much more than that. The breakdown of L-malic acid to L-lactic acid imparts microbiological stability, while the formation of various metabolic end products influences sensory aspects of the wine. The overall reduction in acid, together with the accompanying increase in pH, results in better, "softer" and "rounder" wines with increased body (Bauer and Dicks 2004, Davis et al. 1985, Kunkee 1967, Lallemand 1999 to 2004, Rankine 1990, and Wibowo et al. 1985).

In the past, MLF was, to a great extent, conducted by indigenous population(s) of wine lactic acid bacteria (LAB) with varying degrees of success. The MLF often did not start, was delayed, or was conducted by undesired indigenous strains of LAB, all of which are capable of altering the sensory characteristics of the wine. When MLF is delayed, starts then slows, or stops completely, a wine of lower quality is the usual result.

The pH of the media is capable of drastically influencing the MLF itself as low pH inhibits the growth of the wine LAB *Oenococcus oeni,* which is the organism of choice to conduct MLF under acidic conditions. When the pH is too high, heterofermentative *Lactobacillus* strains and *Pediococcus* are capable of growing, and this is an undesirable scenario.

In recent years, inducing MLF by inoculation with commercially available, very reliable wine LAB cultures has become the norm. These cultures not only offer a more reliable and less uncertain MLF, they also significantly impact the sensory aspect of wines. These bacterial strains are selected according to very strict criteria and are able to function under extremely harsh conditions. They have a defined positive impact on the sensory profile of wines and contribute to increased mouthfeel and complexity (Bauer and Dicks 2004, Davis et al. 1985, Henick-Kling and Acree 1998, Rankine 1990, and Wibowo et al. 1985). The reliability and effectiveness of selected wine LAB cultures have improved considerably in the past decade, and numerous factors are involved in their success. Several factors, some of which are well known and others less well known, as well as their interactions and additive effects, play a role in the successful course of MLF. These factors will be discussed to better understand the process and the management of MLF.

WELL-KNOWN FACTORS THAT AFFECT MALOLACTIC FERMENTATION

The best understood factors that govern successful MLF are SO_2, pH, alcohol and temperature. For MLF to be successful, the values of these chemical parameters must correspond to those that allow the bacterial cultures to function successfully. A favourable level of any one of these components may compensate for an unfavourable level of one or several of the others. It is important to remember these factors function synergistically, i.e., their actions together have a greater total effect than the sum of their individual actions. Molecular SO_2 is effective as a bacterial preservative, and a well-known synergistic effect is the impact of pH on the level of molecular SO_2 (table 1). The lethal level of molecular SO_2 for most wine LAB is low (0.3 mg/L), but it is possible that certain selected wine LAB strains could have a better resistance to molecular SO_2. Depending on the pH of the juice/wine, the amount of molecular SO_2 is between 1% and 7% of the free SO_2 content. The molecular SO_2 increases with a decrease in pH and an increase in temperature and/or alcohol.

Table 1. pH and molecular SO_2

MOLECULAR SO_2 AT DIFFERENT ETHANOL LEVELS								
Free SO_2 (mg/L)	Molecular SO_2 (mg/L) 18° – ethanol at 8% v/v				MolecularSO_2 (mg/L) 18° – ethanol at 13% v/v			
	pH 3.0	pH 3.2	pH 3.4	pH 3.6	pH 3.0	pH 3.2	pH 3.4	pH 3.6
5	0.23	0,14	0.09	0.06	0.41	0.26	0.16	0.10
8	0.37	0.23	0.15	0.09	0.66	0.42	0.26	0.17
10	0.46	0.29	0.18	0.11	0.83	0.52	0.33	0.21
15	0.69	0.43	0.27	0.17	1.24	0.78	0.49	0.31

Wine LAB are susceptible to molecular SO_2, which means that SO_2 additions to the product must be performed judiciously, especially when the pH is low. As the level of molecular SO_2 depends on many factors, it may be wise to calculate the molecular SO_2 level prior to inoculation with wine LAB. Interactive tables are available (at etslabs.com, for example) for this purpose.

In an effort to assist the winemaking community, Lallemand has developed a scoring system to assess the MLF potential of a wine. Each relevant condition is assigned a numerical score, and the summation score indicates whether MLF is likely to be easy or difficult (table 2).

Table 2. Scorecard for determining the ease of malolactic fermentation

CONDITION	1 point each	2 points each	8 points each	10 points each		Score
Alcohol (% vol)	<13	13 - 15	15 - 17	>17		
pH	>3.4	3.1 - 3.4	2.9 - 3.1	<2.9		
Free SO_2 (mg/L)	<8	8 - 12	12 - 15	>15		
Total SO_2 (mg/L)	<30	30 - 40	40 - 60	>60		
Temperature (°C)	18 - 22	14 - 18 or 22 - 24	10 - 14 or 24 - 29	<10 or >29		
Yeast's nutritional needs	Low	Medium	High	Very high		
Ease of alcoholic fermentation	No problems	Transient yeast stress	Sluggish / stuck AF	Prolonged yeast contact		
Initial level of malic acid (g/L)	2 - 4	4 - 5 or 1 - 2	5 - 7 or 0.5 - 1	>7 or <0.5		
Maximum AF rate (maximum loss of Brix/day)	<2	2 - 4	4 - 6	>6		
Note: Other, currently less-well-known factors that are not considered in this scorecard may include the level of dissolved oxygen, polyphenolic content, lees compacting, pesticide residues, etc.						
Total score for the ease of malolactic fermentation:						

Total scores are interpreted in the following manner.

Total scores under 13 reflect favourable MLF conditions:

- Inoculate rapidly with selected bacteria
- *Brettanomyces* and contaminating bacteria may be present and may grow quickly

Total scores between 13 and 22 reflect less favourable MLF conditions:

- Choose a selected wine LAB strain adapted to the most limiting parameter
- A specific bacteria nutrient may be necessary

Total scores between 23 and 40 reflect difficult MLF conditions:

- Give preference to a selected wine LAB strain adapted to the specific wine conditions
- Consider a step to acclimatize the wine LAB to the juice/wine conditions
- Adjust temperature to 18° to 20°C (64° to 68°F), supply bacteria nutrition and add yeast cell wall preparations 24 hours prior to inoculation with wine LAB

Total scores over 40 reflect extremely difficult MLF conditions:

- The wine, as it is, will not undergo MLF

The Scorecard for Determining the Ease of Malolactic Fermentation is a very useful tool, but it is difficult to assign exact values to each category. To ensure the best possible degree of success, it is imperative to abide by the recommendations of the producers of selected wine LAB starter cultures (Bauer and Dicks 2004, Davis et al. 1985, Ribéreau-Gayon et al. 1998, and Vivas et al. 2000).

There are two basic considerations when choosing a selected wine LAB starter culture: the culture's compatibility with the wine environment and its sensory attributes. Tolerance to the four main environmental parameters – pH, free and total SO_2, alcohol level and temperature – is the most important consideration when selecting the proper wine LAB strain to ensure successful MLF.

In some cases, it is very difficult to produce a wine whose analyses conform to these general parameters. New World red wines made from grapes harvested at very high maturity and with subsequently high alcohol levels are a typical example, as are low pH wines from cold climate regions, where levels of molecular SO_2 play an important role.

(Complete text available in printed version of the book)

DETERMINING WHEN TO ADD THE SELECTED WINE LACTIC ACID BACTERIAS

SIBYLLE KRIEGER-WEBER, PH.D., AND ANTHONY SILVANO

Malolactic fermentation (MLF) occurs in wine as the result of the metabolic activity of wine lactic acid bacteria (LAB). MLF reduces wine acidity and modifies wine flavour, both of which are considered to be beneficial to wine quality. Additionally, the use of selected strains of wine lactic acid bacteria (LAB) allows for better control of the timeframe of L-malic acid degradation. Sensory studies show that flavour compounds produced by wine LAB impart recognizable changes to the flavour characteristics of wine (Laurent et al. 1994, Costello, Francis et al. 2012, Costello, Siebert et al. 2012, and Knoll et al. 2011). Several studies show that different strains of wine LAB will have different sensory effects in wines (Laurent et al. 1994, Henick-Kling et al. 1994, Martineau and Henick-Kling 1995, Müller Botti 1996, and Rosi et al. 1998). The timing of the bacterial addition and the number of cells in the wine after inoculation influence the sensory profile (Abrahamse and Bartowsky 2012).

TIMING OF SELECTED WINE LACTIC ACID BACTERIA INOCULATION

Although not recommended, MLF can be conducted by indigenous wine LAB present in the winery infrastructure, which may occur during alcoholic fermentation (AF) or immediately after its completion. Traditionally, when selected cultures of known wine LAB are used, inoculation is performed at the completion of AF. Beelman and Kunkee (1985) explored the possibility of inoculating wine LAB into juice along with the yeast used to conduct AF. Current thinking identifies the following times during wine production when selected wine LAB can be added (figure 1).

- Co-inoculation with yeast and selected wine LAB
  - Selected wine LAB added 24 to 48 hours after yeast addition (48 to 72 hours if 80 to 100 ppm of SO_2 is added at crushing)
- Early inoculation
  - Selected wine LAB added during active AF or at an approximate density of 1040/1030 (8°/10°Brix)
- Post-alcoholic fermentation
  - At the end of, or just after, completion of AF
- Delayed inoculation
  - 2 to 6 months after completion of AF

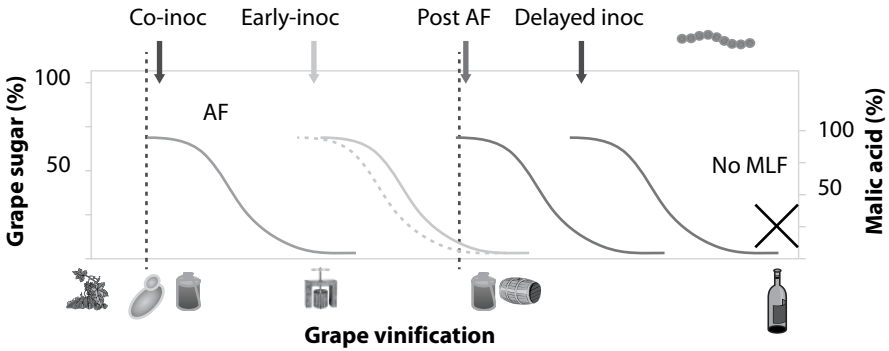

Figure 1. Inoculation regimes for selected wine lactic acid bacteria – Adapted from Bartowsky, AWRI, 2010

CO-INOCULATION WITH SELECTED YEAST AND SELECTED WINE LACTIC ACID BACTERIA

Before 2003, researchers at the Université de Bordeaux recommended making the wine LAB addition only after the completion of AF. They felt this timing would avoid the production of acetic acid and D-lactic acid, compounds derived from the heterofermentative carbohydrate metabolism of LAB (Ribéreau-Gayon et al. 1975). They proposed that wine LAB added at earlier points during AF may result in slow or stuck yeast fermentation, or result in MLF inhibition due to yeast antagonism. To date, none of these concerns have been observed when both AF and MLF have been properly managed.

The inoculation of selected wine LAB into juice along with yeast was proposed because it was felt nutrient availability would be enhanced, and the absence of alcohol would allow wine LAB to better acclimatize to environmental conditions and grow more vigorously. Beelman and Kunkee (1985) showed that MLF in the presence of fermentable sugars does not necessarily lead to the production of excessive amounts of acetic acid, as long as yeast fermentation starts promptly and goes to completion (Krieger 2002, and Sieczkowski 2004). King and Beelman (1986) suggested the growth of *Leuconostoc oenos (Oenococcus oeni*) PSU-1 during AF in a model grape juice system may be delayed by the production of yeast-derived toxic compounds other than ethanol and SO_2. When they compared the bacterial growth curves of a pure culture and a mixed culture with yeast, they found the presence of rapidly growing yeast was antagonistic toward bacterial development (figure 2). They attributed the inhibition of bacterial growth to the presence of yeast metabolites and/or the removal by yeast of substances important to bacterial nutrition. The data in figure 2 shows the point at which bacteria transition from a lag phase to a logarithmic growth phase is coincident with the onset of yeast death. This may be the result of yeast autolysis returning essential nutrients to the system. Yeast growth in pure and mixed cultures was unaffected by the presence of bacteria. An accelerated yeast death rate in mixed culture was observed when rapid bacterial growth occurred.

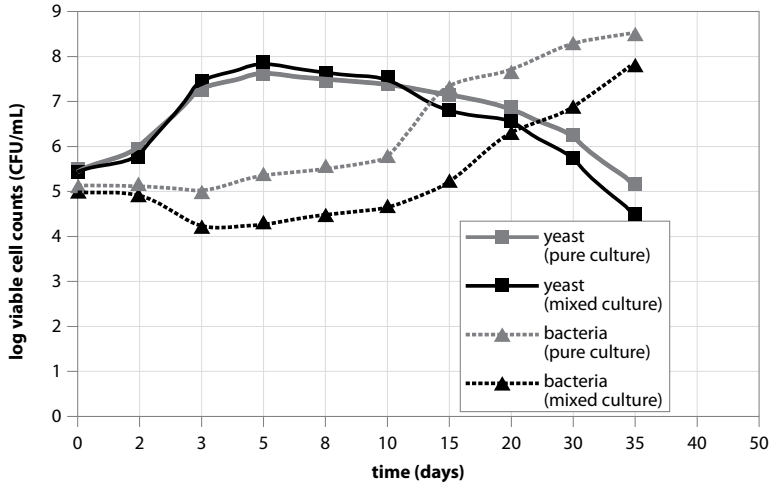

Figure 2. Growth of wine yeast and wine lactic acid bacteria in pure and mixed cultures

When a stuck AF occurs, the presence of indigenous LAB is usually associated with the problem. A recent publication (Jarosz et al. 2014), states that a microbial biochemical communication system was detected, which may explain the observation. This paper recounts that a group of scientists detected prions (abnormally shaped proteins capable of self-replication)

capable of directing yeast to process carbon sources other than glucose. This results in a dramatic slowing of fermentation, with its eventual cessation (Bisson 2014). Using a mechanism that does not alter yeast DNA, the prions associated with bacteria direct yeast to switch to food sources other than sugars. The LAB responsible are heterofermentative *Lactobacillus kunkeei* and *Lactobacillus naglei*, and homofermentative *Pediococcus damnosus*. *O. oeni* has not been reported as a causative organism. Edwards et al. (1999) reported acetic acid production by *L. kunkeei* as being partially responsible for yeast inhibition, but suggested that additional inhibitory mechanisms may be probable.

Early work in France found that yeast growth was negatively affected by the growth of wine LAB, leading to the production of excessive amounts of volatile acidity (VA) (Lafon-Lafourcade and Ribéreau-Gayon 1984). Radler (1963) described the three phases of wine LAB growth, as shown in figure 3. During growth in Phase I, small amounts of acetic acid and D-lactic acid can be produced from the metabolism of carbohydrates. In Phase II, when cell numbers exceed 10^6 CFU/mL, L-malic acid degradation begins with L-lactic acid formation, but with no acetic acid production. Phase III is characterized by the degradation of citric acid and sugars, accompanied by an increase in acetic acid. The bacteria will begin to consume sugars only when the degradation of organic acids is complete. L-malic acid will be the first consumed, followed by citric, fumaric and other acids (Krieger 1989). The degradation of sugars at this point under high pH conditions (pH >3.4) will result in a significant increase in VA.

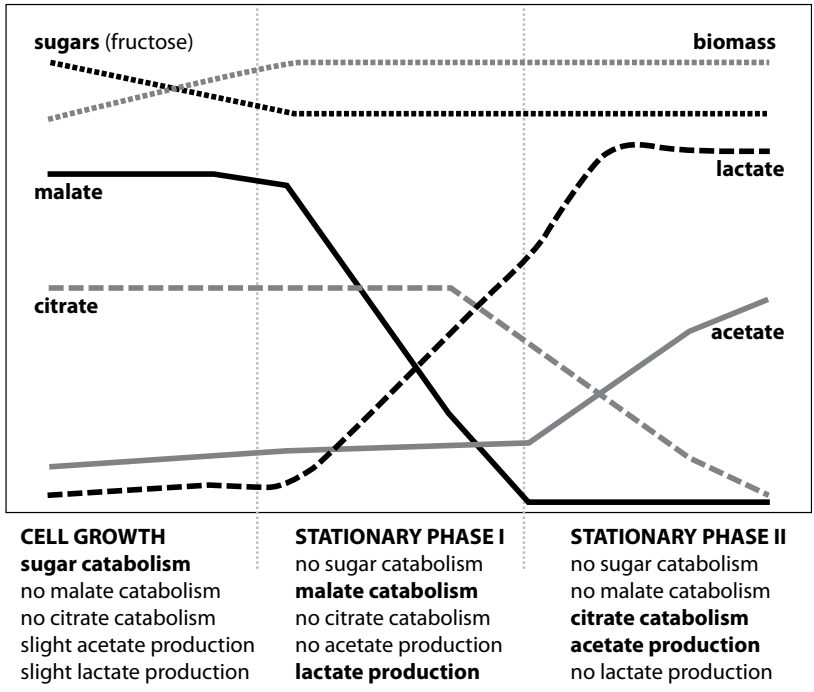

Figure 3. Metabolism of sugars and organic acids during malolactic fermentation in wine

Differences in pH drastically affect the metabolic behaviour of wine LAB. At pH 3.5 and above, wine LAB are more likely to decompose sugars and citric acid (Dharmadhikari 2014), which may lead to higher VA levels. Below pH 3.5, *O. oeni* shows very little affinity for sugars, but prefers malic acid, followed by citric acid and fumaric acid. Citric acid degradation is strain dependent. Some selected wine LAB strains will begin to degrade it at the midpoint of MLF, while others will start only after complete depletion of L-malic acid. Experiments conducted by Lallemand confirm that acetic acid will not be produced during the growth of wine LAB and active MLF. In these experiments, acetic acid production was observed only when half of the L-malic acid was degraded and the bacteria began to utilize citric acid. A direct relationship between citric acid degradation and an increase in acetic acid concentration was demonstrated (Krieger 2002). Knoll et al. (2012) studied the impact of the following MLF inoculation scenarios on Riesling aroma:

- Selected wine LAB addition 24 hours after yeast addition (co-inoculation)
- Selected wine LAB addition at 40% completion of AF
- Selected wine LAB addition at 60% completion of AF

- Selected wine LAB addition at completion of AF

Trials conducted using co-inoculation vs. selected wine LAB addition at the completion of AF show no difference in the final acetic acid concentration (table 1).

Table 1. Concentration (g/L) of acetic acid and citric acid after malolactic fermentation – Adapted from Knoll et al. 2012

	VP41				PN4			
Timing of inoculation	24 hr	40%	60%	End AF	24 hr	40%	60%	End AF
Acetic acid	0.61	0.61	0.66	0.73	0.66	0.66	0.68	0.77
Citric acid	0.14	0.15	0.17	0.11	n.q.	n.q.	n.q.	n.q.
	n.q.: Not quantifiable (limit of quantification 0.1 g L-1)							

CO-INOCULATION WHEN pH IS BELOW 3.5

Lallemand, in collaboration with Massey University in New Zealand, made wine using one selected yeast strain and two selected wine LAB strains. For each yeast/bacteria combination, wine LAB were inoculated into the must either 24 or 48 hours after yeast addition – the co-inoculation technique – or at completion of AF – the sequential technique. Chardonnay grapes were pressed without SO_2 addition, and the must was cold settled at 4°C for 24 hours, then racked and supplemented with 300 mg/L of diammonium phosphate. Vinifications were carried out in triplicate. The results presented in table 2 show that sequential inoculation resulted in prolonged MLF as compared to co-inoculation (Jussier et al. 2006).

Table 2. Malolactic fermentation delay vs. inoculation sequence

	Co-inoculation AF/MLF	Sequential AF/MLF
ML starter culture A	26 days	74 days (malic acid remained)
ML starter culture B	20 days	68 days

(Complete text available in printed version of the book.)

THE PROGRESS, REALITY AND FUTURE OF SELECTED WINE LACTIC ACID BACTERIA CULTURES

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WHAT WE HAVE LEARNED OVER THE PAST THREE DECADES

The role of malolactic fermentation (MLF) in red wines and many white wines is constantly being verified. Initially recognized as a way to deacidify wine, MLF was adopted because it imparted a degree of microbiological stability to the product and because it enriched sensory attributes of wines. Gradually, oenologists and winemakers paid attention to the sensory impacts of oenological practices in general and the contribution of microbiological practices in particular. Tasting, which has become increasingly important in the evaluation of wines, clearly confirms the contribution of MLF to the sensory characteristics of both red and white wines. In certain regions, winegrowing practices and increased grape maturity have resulted in the gradual reduction of the concentration of malic acid in musts. Nevertheless, MLF is useful, not necessarily for its deacidification function, but for its impact on the sensory composition of wines.

In the 1970s, producers were very interested in the role of yeast in winemaking. Starter cultures made from active dry yeast (ADY) are easy to use and highly diverse. They have been carefully studied for their oenological properties, their fermentation capacities and their in-

fluence on taste and aroma. Success was much slower for selected wine lactic acid bacteria (LAB) starter cultures due to the very nature of the organism, their physiology and their sensitivity to environmental factors. Performance failures of wine LAB starter cultures in the 1980s kept them from being widely accepted for use. The selectors and producers of starter cultures have expanded their research efforts, resulting in progress that instills trust, interest and demand on the part of users. Advances in microbiological processes have resulted in a need to understand the role of each micro-organism in the winery as both academic oenologists and winemakers are interested in the tangible aspects of the microbiology of winemaking. Information disseminated in seminars and trade publications has furthered the practice of using malolactic starter cultures, a process that has overcome its difficult beginnings and is now very beneficial.

Winemaking is a microbiological process involving a very complex system. The nature of the micro-organisms that inhabit grape must, along with the must's chemical composition and temperature, influences the parameters on which wine quality depends. Yeast and bacteria populations in wine are varied and variable. During alcoholic fermentation (AF), different yeast species successively dominate, while others remain in the minority or disappear. The four fundamental parameters of pH, alcohol, temperature and CO_2 determine the evolution of indigenous microbial populations, which may be monitored by winemakers from the beginning to the end of AF. It is known that interactions among micro-organisms are the key factors in fermentation, but these interactions are difficult to evaluate. The use of yeast starter cultures that massively increase the population is a convenient and sure way to master the microbiological composition of the system. The addition of yeasts has become a classic step in winemaking, and has become more refined as there is now a very wide choice of strains available on the market. Currently, this step is becoming more complex with the arrival of non-*Saccharomyces* yeast species. The addition of commercially available wine LAB is becoming more established as the available starter cultures become more reliable.

Due to the efforts of technical centres, research centres, industrial R&D departments and end users, considerable progress has been made in the functionality of commercially available wine LAB starter cultures. The major problem to overcome was the survival and growth of the bacteria inoculated into wine as their population must be sufficiently high to ensure MLF. Wine LAB will not degrade L-malic acid unless they are viable, and viability is necessary for maintaining essential cell membrane functions. Wine is a medium not conducive to microbial growth because it contains relatively high levels of acid, ethanol, fatty acids, polyphenols and other compounds. Indigenous microflora, on the other hand, are well adapted to these conditions, but the adaptation may be lost. If we understand the reasons for the loss of adaptation, how it can be prevented and how resistance can be reacquired, this knowledge could be

applied to commercially available wine LAB starter cultures. The subject has been examined by academia as well as by industry.

The ability of wine yeast or wine LAB to tolerate stress is a key element of the process used to select new wine LAB strains. The degree of variability between strains is high, necessitating screening of broad numbers of collections. Selection is the key to success, but the development and mastery of the industrial growth process, harvesting techniques and drying methods are of equal importance. Pilot wine LAB trials in the winery are now more numerous. As a result, influences exerted by the chemical characteristics of wine are becoming better understood and the sensory contributions of wine LAB have become as important as the ability to degrade L-malic acid. The sensory contributions of the isolates are now a part of the basic criteria used for selecting new wine LAB.

Scientists are examining the bacteria's toxicity tolerance mechanisms. The plasma membrane is considered to be the first line of defense because neither survival nor metabolic activity is possible if the barrier between the cytoplasm and the surrounding media is not intact (Garbay and Lonvaud-Funel 1996). One of the proteins associated with the cell membrane, Lo18, has been extensively studied. It is involved in stress response, maintaining membrane integrity through interactions with phospholipids (Coucheney et al. 2005). Other cytosolic stress proteins have also been studied, and the results have provided direction for further research.

Along with the choice of the proper selected wine LAB strain to use, the timing of the addition of the MLF starter culture is crucial. Commercial wine LAB starter cultures can be adapted to wine by inoculating them into the acidic but low alcohol must at the start of, or 24 to 48 hours into, the AF. The term "co-inoculation" is used to describe this process, but the yeast and bacteria are rarely added at the same time. Selected wine LAB develop in the fermenting must where they reactivate tolerance mechanisms that facilitate their growth in wine. Numerous wine producers have tried and adopted this practice as the time required to completely degrade L-malic acid is greatly reduced compared to sequential inoculation, which is the addition of selected wine LAB after AF has completed. Co-inoculation is particularly advantageous for low pH wines as shown by Knoll et al. (2012). They showed that in a Riesling must with a pH of 3.1, L-malic acid was degraded from 25 to 50 days sooner than it was when sequential inoculation was used, and that fruity aroma esters were more abundant. The sensory impact of co-inoculation has been demonstrated in other trials as well, especially with Merlot, where it significantly influences the aroma profile. Generally, the fruity and lactic characters are affected the most. The fruity notes can be boosted either by an increase in ester concentration or by a decrease in lactic notes. Lactic notes are decreased either because esters are degraded, or because a lactic and smoky mask has developed (Antalick et al. 2013). Co-inoculation

modifies the wine's aroma profile, but it is difficult to identify a general trend for two reasons: first, the wine LAB malolactic enzyme activities responsible are linked to overall bacterial metabolism, which is influenced by interactions with the yeast and the population ratio, and second, substrates available to wine LAB depend on the simultaneous influence of yeast and bacteria on final wine composition, notably for the taste and aroma.

GENOMICS, A NEW APPROACH

Molecular biology made its appearance in oenology in the early 1990s. Subsequent advances in equipment and methodology have permitted research that was previously not possible. The first application of genetic techniques to oenology was the precise and reliable typing of yeast and bacteria strains. The methodology initially used probed hybridization and the genetic profiles of entire or hydrolyzed genomic DNA obtained by a variety of electrophoretic techniques. Until the advent of newer genetic techniques, it was extremely difficult to distinguish one microbiological strain from another of the same species, unless it had clearly different biochemical characteristics. Producers of wine LAB cultures took advantage of strain typing to classify the organisms in culture collections and to control their production. Academic oenologists, as well as production winemakers, used strain typing to control the implantation of inoculated organisms. Polymerase chain reaction (PCR) protocols were developed to detect *Brettanomyces bruxellensis* and other spoilage bacteria, and are specific, sensitive, reliable and affordable.

Future research into the microbiology of wine will be through the study of microbial genomes in association with physiological and metabolic studies under conditions reflective of wine parameters. For selected wine LAB starter cultures, the crucial question deals with their survival after inoculation into wine. Research in the 1990s clearly identified the deleterious effects of low pH, alcohol, fatty acids and other toxic molecules on the wine LAB cell membrane, whether initially present as must components or synthesized during AF. Definitive genetic studies to explain these observations, however, were hampered as the required techniques were not yet available.

The early 2000s marked a turning point in the study of *Oenococcus oeni* as sequencing of the entire genome became possible. The next step was comparing genomes of various strains and then linking them to known physical characteristics of the strains. Today, there are 23 listed *Oenococcus oeni* genomes in the international sequencing bank at the National Center for Biotechnology Information (NCBI). Computer data processing has permitted a better understanding of the genetic origin of properties associated with strains of *O. oeni*…. *(Complete text available in printed version of the book.)*

In an effort to compile the latest usable information regarding malolactic fermentation, Lallemand published Malolactic Fermentation in Wine - *Understanding the Science and the Practice* in 2005. This addition is an update to that publication with new and relevant information. We intend it to be a compendium of both scientific and applied information of practical use to winemakers from all geographic areas and wine growing regions. It is the desire and intention of the authors to supply the industry with information winemaking professionals can use in the pursuit and furtherance of their art.

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