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Leuconostoc oenos and malolactic fermentation in wine: a review

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This review article summarizes the state of the art on *Leuconostoc oenos*, the bacteria responsible for malolactic fermentation in wine. Both basic and practical aspects related to the metabolism of this microorganism and malolactic fermentation in general are critically reviewed. The former examines the role of genetics for the identification and classification of *L. oenos* and energetic mechanisms on solute transport (malic and lactic acid). The latter includes practical information on biomass production, optimal growth conditions and stress factors, which are important in growth optimization of malolactic starter cultures. Extensive data and references on the effect of malolactic fermentation on wine composition and sensory analysis are also included.

Keywords: malolactic fermentation; *Leuconostoc oenos*; wine; review

Introduction

The malolactic fermentation (MLF), a so-called secondary fermentation, results from the metabolism of certain lactic acid bacteria in wine and consists in the conversion of L-(−)-malate to L-(+)-lactate and CO_2. Basically, the two acidic groups of malate are replaced with only one acidic group present in lactate which results in a decrease in acidity of the wine. Different bacteria genera (eg, *Leuconostoc*, *Pediococcus* and *Lactobacillus*) have been reported to carry out MLF in wine produced worldwide [12,32,46,57,67,70,110,111,133,151]. Among them *Leuconostoc oenos*, more recently reclassified as *Oenococcus oeni* [26], is recognized as the bacterium most tolerant to the wine conditions, such as low pH, high SO_2 and alcohol content [73,142,146]. MLF usually occur in wine after the alcoholic fermentation when the bacterial population is about 10^6 CFU ml^{-1}. MLF in wine is desirable for three reasons: (i) to decrease the acidity; (ii) to enhance the organoleptic characteristic; and (iii) to increase the microbiological stability of wine. However, MLF is not favorable for all wines. In fact, in warmer areas grapes tend to be less acid and a further decrease in acidity by MLF may be deleterious for the sensory properties and biological stability of the wine [21,58,60,74,75,88,120]. The process of MLF in wine is only partially understood and difficult to predict. Therefore, an improved knowledge of MLF is essential to control (stimulate or arrest) this important process. The use of immobilized cells/enzymes of *L. oenos* for achieving the MLF has not been addressed in this review [14,29,30,37,39,44,145].

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Genetics

Several strains of *L. oenos* may perform MLF in wine, therefore an easy, sensitive and accurate method for their identification and characterization in starter cultures is essential. In the 1980s several attempts were made at typing *L. oenos* strains using a single method based on bacterial differences in carbohydrate fermentation. Plasmid profiles (unfortunately only a few of the strains of *L. oenos* contain plasmids) or phage sensitivity patterns were only partially successful [58]. Recently, more reliable tests have been proposed to monitor individual strains during MLF. Transverse alternating field electrophoresis (TAFE) [20,80] and pulsed-field gel electrophoresis (PFGE) patterns of digested chromosomal DNA [71,139] have been used to differentiate closely related strains of *L. oenos* and also to differentiate this bacterium from other oenological lactic acid bacteria belonging to *Leuconostoc* spp. Each strain displays a characteristic restriction pattern suggesting that *L. oenos* sp consists of a genetically heterogeneous collection of strains. A two-step approach, having ribosomal gene restriction fragment length polymorphism (RFLP) associated with SDS-PAGE total DNA restriction profiles, has been proposed [147]. The ribopatterns among *L. oenos* strains were easily distinguished from other phylogenetic tree adjacent species, such as *Leuconostoc* and *Weisella* (these two species being in the other two branches of the phylogenetic tree as confirmed by 16S and 23 S rRNA sequencing studies) [15,25,98,99].

In view of these results Dicks *et al* [26] proposed a reclassification of *L. oenos* into a new genus as *Oenococcus oeni*. The ribopattern method would be capable of distinguishing strains belonging to species different from *L. oenos*, while the total DNA restriction profiles could provide a useful tool for typing of *L. oenos* strains. More recently, a tRNA-Ala gene has been identified in the intergenic spacer located between 16S and 23S rRNA genes in *L. oenos*. The highly conserved 18-nucleotide tRNAI

sequence in combination with non-specific oligonucleotides has been used for PCR of genomic DNA which allowed strain differentiation in *L. oenos* [82]. For other authors [153], the comparison of 16S–23S intergenic spacer region (ISR) sequences, associated with random amplified polymorphic DNA (RAPD), demonstrated that *L. oenos* is phylogenetically a very homogenous species, different from the genus *Leuconostoc*. 16S-23S ISR-RFLP analysis permitted attribution of strains of *Leuconostoc* to the species *oenos*. Moreover the RAPD profiles of *L. oenos* were strain-specific and discerned two main groups of strains [154,155]. A reliable method based on DNA-DNA hybridization has been used for detection of several species of lactic acid bacteria [25,27,89]. This method is species-specific but not strain-specific.

A three-component MLF system has been proposed for *L. plantarum* [108] and *L. oenos* [18] including: (1) malate transport; (2) malolactic enzyme; and (3) lactate transport. Henick-Kling [58] isolated spontaneous mutants of *L. oenos* affected in transport of malate but not by malolactic enzyme. However, only recently the genes encoding malolactic enzyme (*malA*) and malate permease (*malP*) have been cloned and sequenced [77]. Significant levels of malolactic activity were observed when expressing the *mleA* gene in *E. coli* and *S. cerevisiae*. Complete characterization of the *mle*L locus came from the same authors [78]. Northern blots suggested an operon structure harboring *mleA* and *mleP* genes with a unique transcription start site. In addition to the upstream of *mleA*, they identified an open reading frame, called by the authors *mleR*-like gene, which encoded a polypeptide belonging to the LysR-type regulatory protein family identified in other bacteria [121,150]. The confirmation of a *malR*-like gene involvement in *mle* operon expression is underway. After sequencing of the *mleA* gene in *L. oenos*, a species-specific PCR has been proposed for the identification of this bacteria in wine and must [153]. In addition, specific polyclonal antibodies against malolactic enzyme (MLE) of *L. oenos* have become available, and have been used against the MLE gene expressed in *E. coli* [79].

The histidine decarboxylase gene (HDC) from *L. oenos* has been cloned recently [16]. Because of the negative effects of amines in wine, these results can be a useful tool for selecting better strains which are used as starter cultures.

Bioenergetics

The pathway of MLF includes the uptake of L-malate, its decarboxylation to L-lactic acid and CO_2, and excretion of the end products (including a proton). The decarboxylation reaction is catalyzed by the malolactic enzyme (L-malate:NAD^+ carboxy lyase) (IUC number 1.1.1.38) in the presence of NAD and Mn^{2+} [75,88,102,103,136]. This reaction does not yield energy-rich phosphate bond intermediates directly, however the electrochemical energy can be conserved via an indirect electrical potential ($\Delta\Psi$). As a proton is consumed in the decarboxylation reaction the internal pH increases. Alkalinization of the cytoplasm results in creation of a chemical potential of protons across the membrane (ΔpH) that, together with the $\Delta\Psi$, forms the proton motive force (PMF) across the cytoplasmic membrane (chemiosmotic mechanism). The relative contribution of $\Delta\Psi$ and ΔpH depends on the mechanism of transport systems: uniporters (transit of one solute across the cytoplasmic membrane), symporters (combined translocation of two or more solutes in the same direction) and antiporters (associative transport of a solute in one direction to the translocation of another solute in the opposite direction) [28,72,115].

For the first time a model has been presented for *L. oenos* in which the PMF generated by efflux of L-lactic acid creates a ΔpH able to drive L-malate transport and generate ATP via the membrane ATPases. Despite the fact that *L. oenos* cannot grow with L-malic acid as sole carbon source, MLF supplies the cell with additional metabolic energy (ATP), which probably is responsible for a stimulatory effect during the early stage of growth [18]. Loubierre *et al* [92] using batch cultures of *L. oenos* at pH 5.0, reported improved growth rates when both glucose and malate were used as energy sources compared with glucose alone. They attributed this result to a chemiosmotic transport mechanism rather than a proton consumption by the malolactic enzyme. Results indicated that *L. oenos* takes up malate by a L-malateH^-/H^+ symport (although a low-affinity L-malateH^- uniport is also implicated) and liberation (efflux) of lactate by electroneutral lactate$^-/H^+$ symport. This process occurred with a constant stoichiometry.

A second model for L-malate uptake was proposed by Tourdot-Maréchal *et al* [140]. A study with a mutant strain Lo84.13, unable to decarboxylate L-malic acid, indicated the presence of two simultaneous uptake mechanisms for L-malic acid: a low-affinity L-malateH^- uniport and a passive diffusion of undissociated L-malic acid. The relative contribution was found to be ΔpH-dependent. At pH $>$ 4.5, malate transport was carrier-mediated, while at pH 3.2 about 64% of L-malic acid was found undissociated and passive diffusion represented more than 50% of the total L-malic acid uptake (diffusion constant, $K_D = 0.1\ s^{-1}$). The same authors [141] confirmed the possibility of a ΔpH-dependent transport of L-malate via membrane vesicles from strain Lo107.

Later Salema *et al* [128], studied L-malate transport in membrane vescicles from strain Lo84.13 and proposed a third model in which L-malate was taken up in the L-malateH^- form by a uniport mechanism operating at low L-malate concentration and at pH 3.0–5.6. In whole cells, the driving force for L-malateH^- uptake was found to be the L-malateH^- concentration gradient which resulted from decarboxylation of L-malate inside the cell. A second transport mechanism was observed only at L-malate concentrations above 1 mM; this component could either passively diffuse or follow carrier-mediated transport with low affinity (apparent $K_m > 10$ mM).

Under the conditions of MLF in wine (pH $\cong$ 3.5), the chemical gradient of L-lactic acid (pK_a 3.8) is low. Lactic acid leaves the cell by passive diffusion. It appears that different species of malolactic bacteria possess different transport systems. Salema *et al* [128] suggested a uniport mechanism for L-malateH^- uptake in bacteria such as *L. oenos* that ferment L-malate at relatively low pH. The same authors [129] later confirmed the uniport mechanism for

transport of L-malateH$^-$ in resting cell cultures of commercial strain GM (which generate a $\Delta\Psi$ between –88 mV and –170 mV), and suggested that the rate of MLF is rate-limited by L-malic acid uptake and controlled by the $\Delta\Psi$ generated. At pH 3.5 the rate of ATP synthesis was highest, implying a lower $\Delta\Psi$ and a maximal external L-malateH$^-$ concentration. MLF allows *L. oenos* to take up nutrients by the chemiosmotic mechanism and maintain a suitable pH for enzymatic activity and cell growth, especially at lower pH values. Further studies showed that citrate transport in strain GM is regulated by the same uniport mechanism observed for malate [117].

Biomass production and malolactic activity

Lactic acid bacteria cannot grow with L-malic acid as a unique carbon source, therefore these microorganisms need an additional energy source, such as residual fermentable sugars, ie glucose or fructose [84] or amino acids such as arginine, to allow cell growth [87]. The role of citric acid should also be taken into account [65]. Substrate co-fermentation by *L. oenos* largely depends on the strain used as well as on the environmental conditions (eg substrate, pH, temperature). In a study conducted on Cabernet Sauvignon, Pinot Noir and Chardonnay wines, using 11 commercial strains of *L. oenos*, malic and citric acid were co-metabolized. The rate of malate utilization was dependent on the type of wine [96]. When D-glucose, L-malic and citric acid were consumed simultaneously at pH 4.8, L-malic acid was metabolized by strain *M* at a higher rate than glucose and citric acid [127]. It appears that when *L. oenos* is allowed to catabolize malic acid before glucose, the consumption rate of sugar increases. In particular, *L. oenos* seems to prefer malate over glucose and citrate as an energy source at low pH [4]. Pimentel *et al* [113] reported that three strains of *L. oenos*, isolated from Portuguese wines, metabolized malate before glucose except at high pH (4.0 and 4.5). In this case, citric acid repressed the consumption of fructose and the consequent formation of mannitol, while stimulating co-utilization of glucose and production of acetate. It seems that sugars are not metabolized at low pH [60]; in this condition the MLF is able to increase the pH to values compatible with cell growth (in fact, cell growth is not necessary to accomplish MLF) [38]. Mixed-substrates fermentation stimulated cell growth of *L. oenos* in a model solution at pH 5.0. The specific growth rate (μ_{max}) increased from 0.05 to 0.087 and 0.14 h^{-1}, using glucose, glucose-citrate and glucose-fructose substrates, respectively. These results were correlated with an increase in ATP production via the acetate kinase pathway. However, citrate alone did not stimulate microbial growth without the availability of fermentable carbohydrate [130].

Interestingly, during glucose-citrate co-metabolism, the ratio NAD(P)/NAD(P)$^+$ decreased [119]. Miranda *et al* [101] later elucidated the importance of the NAD(P)/NAD(P)$^+$ ratio on the mechanism of sugar-induced inhibition of malolactic activity. Working at pH 3.5, they found glucose (2 mM) able to inhibit MLF by 50% in cell suspensions of strain GM. The maximum inhibitory effect (*ca* 70%) was observed using 5 mM glucose. NADH accumulated during glucose catabolism was responsible for the inhibitory activity on the malolactic enzyme. In the presence of additional electron acceptors (eg, ribose, fructose and citrate), the NAD(P)H/NAD(P)$^+$ ratio decreased, with a concomitant decrease in malolactic inhibition. The lack of inhibition observed in *L. oenos* LoD004 and LoD017 was mainly attributed to a more efficient NAD(P)H disposal, or to inefficient utilization of glucose which prevented accumulation of NADH.

The role of amino acids on MLF is still subject to investigation. Isoleucine, glutamic acid, tryptophan and arginine were essential amino acids for the growth of *L. oenos* strains MC 1, 2 and 4, NCFB 1707, 1823 and 1674 in synthetic medium at pH 5.0. Additional amino acids were also required for optimal bacterial growth. On the other hand, the lack of glycine, phenylalanine, proline and tyrosine limited MLF without affecting growth. An interaction between amino acids and the transport mechanisms involved was postulated [38].

Growth conditions and stress factors

Wine has a complex composition (carbohydrates, pH, SO_2, ethanol, phenolic compounds, fatty acids, amino acids, micronutrients, etc) which vary depending on several conditions, such as cultivar, season, and technology of wine production. Malolactic bacteria have elaborate nutritional requirements [9] and competition for these may inhibit/delay yeast activity during the alcoholic fermentation [33,64]. Lonvaud-Funel [91] suggests that inoculation of must with starter cultures should take place only after the conclusion of the alcoholic fermentation to avoid the increase of wine volatile acidity due to sugar metabolism by *L. oenos*.

The physico-chemical properties that influence microbial growth are well known, mainly: pH, acidity, ethanol and sulfite concentration and temperature [8,23,62,66,151,152]. A central composite design is an effective way to study the interaction among the several factors [50]. Vaillant *et al* [144] studied the effects of 11 physico-chemical parameters (pH, temperature, SO_2, ethanol, glycerol, citrate, malate, tartrate, fructose, glucose, pentoses) on the malolactic activity of three *L. oenos* strains (commercial B1 and B16, and experimental 13A1) using a complex experimental design. Despite the fact that some effects varied with time, ethanol showed the greatest inhibitory effect, followed by pH and SO_2. The increase of L-malic acid concentration prolonged the duration of MLF. Fang and Dalmasso [34] found that sulfur dioxide was more effective against lactic acid bacteria at pH 3.4 than at pH 3.8. Delfini and Morsiani [24] reported that the antiseptic effects of an equal H_2SO_3 concentration were surprisingly stronger at pH 4.0 than pH 3.5. The rate of MLF in wine depends on the bacterial cell density, the specific malolactic activity [104] and the physiological state of the cells. Despite the fact that cells from the exponential phase of growth have the highest specific malolactic activity, these cells were unable to start MLF in wine because of their sensitivity to ethanol. On the contrary, inocula taken from the stationary phase were able to degrade malic acid [47]. Both pH and temperature of incubation were the most important factors affecting bacterial growth, the rates of substrate consumption and the

amount of metabolites produced by three strains of *Leuconostoc oenos* [113].

Spontaneous MLF in wine is often unreliable, the main limitation being nutrient availability and/or the presence of physico-chemical stress factors, including SO_2 (total, free and molecular) [59], lysozyme [43,114], succinic acid or β-phenylethanol [11], nisin [19], must colloids [51] and bacteriophages [1–3,22,56]. The optimization of malolactic starter cultures for MLF induction as well as the achievement of MLF in wine largely depends upon understanding complex interactions between bacteria and the environment. Until recently, commercial preparations required reactivation before inoculation into wine [104]; however, a freeze-dried starter culture is now available for direct inoculation [105]. More information about the mechanisms involved in adaptation to stress conditions and the possible interactions of each wine component on the structure, growth and activity of malolactic bacteria is still required. In fact, the mechanisms allowing *L. oenos* adaptation and growth in wine are only partially understood. Probably, *L. oenos* has several mechanisms to withstand stress conditions. Gallic acid and vanillic acid accelerated the MLF of *L. oenos* IB8413 cultured on Carr medium at pH 4.8. However, only gallic acid increased the rate of decarboxylation, whereas, vanillic acid inhibited microbial growth. The potential role of phenolic compounds as hydrogen acceptors needs further investigation [149].

Strong emphasis has recently been devoted to clarify: (i) the role of fatty acids as a component of the growth medium; and (ii) the effect of the environment on the fatty acid composition of the bacterial cell membrane. Alcohol, temperature and pH can modify the fatty acid composition of the cell membrane of wine lactic acid bacteria. In particular the saturated/unsaturated fatty acids ratio affects the viability of these bacteria [60]. The growth of *Oenococcus oeni* D_{11} (Malolactine O) was stimulated during co-fermentation with *S. cerevisiae* using grape-skin extract as a media component. This result was attributed to the increased level of yeast macromolecules (mainly mannoproteins), and the decreased concentration of inhibitory free fatty acids (especially C_{10} and C_{12}) in the media [52]. The inhibitory effect of fatty acids on malolactic activity and cell growth is concentration- and pH-dependent. Free fatty acids (C_{10} has a $pK_a \cong 4.9$) are present in wine solution ($pH \cong 3.2$) as undissociated molecules. Once inside the bacterial cell, they dissociate with a consequent accumulation of intracellular hydrogen ion and dispersion of the trans-membrane proton gradient, thus inhibiting intracellular enzymes and ΔpH-dependent transport systems. Ethanol (12% v/v) showed an inhibitory effect only on cell growth (malolactic activity was not affected) [10]. The fatty acid composition of the plasma membrane in several *L. oenos* strains varied in response to growth phase and various stress conditions. Ethanol (10% v/v) decreased the unsaturated/saturated fatty acid ratio in the microbial membrane, while addition of wine to the growth medium increased this ratio allowing a direct inoculation and a successful MLF in red wine [40]. The fatty acid composition of *L. oenos* varied not only according to environmental conditions, but was also strain-dependent. In fact, pH greatly modified the fatty acid composition and the degree of unsaturation of strains Lo107 (an acidophilic strain) and Lo8413 (a moderately acidophilic strain), but only slight changes occurred for strain LoATCC 23277 (a less acidophilic strain). At pH 2.9, *L. oenos* Lo107 showed a high level of C19:0 cy-($\omega^{9,10}$) and C19:0 cy-($\omega^{11,12}$) [31].

Garbay and Lonvaud-Funel [41] studied changes in plasma membrane composition of *L. oenos* Lo004D induced by several stress conditions in red wine, such as acidity (pH 3.3 and 3.8), ethanol (10% v/v), toxic fatty acid (C_{10} and C_{12}) and heat (25–60°C). Any type of stress induced an increase in membrane protein concentration (up to five-fold), coupled with a decrease in phospholipid concentration. The lower the phospholipids-to-protein ratio, the better the survival in wine. In particular, a protein of 53 kDa was highly over-expressed. Although no structural function has been investigated, the authors suggest the involvement of a heat-shock or stress proteins. Stress proteins appear to play an important role in acid tolerance of lactic acid bacteria [69]. The ability of *L. oenos* starter cultures to perform MLF in wine was improved after direct inoculation with cells pretreated at 42°C. At this temperature, the synthesis of stress proteins was induced [53]. In *L. oenos*, stresses such as heat (42°C), acid (pH 3.0) or ethanol (12% v/v) induced the expression of an 18-kDa heat shock protein (called LO18) associated with the cytoplasmic membrane [54,68]. *L. oenos* also increases its resistance to SO_2 by adaptation [24]. Guzzo *et al* [55] demonstrated an induction of sulfite tolerance (up to 30 mg L^{-1} total $Na_2S_2O_5$) in *L. oenos* Lo84.13 caused by cell pre-treatment at low pH (3.5) and in the presence of sulfite (15 mg L^{-1} total $Na_2S_2O_5$). Based on the results of expression of gene *hsp*18, these authors suggested that stress protein synthesis and cellular pH homeostasis could be involved in the mechanism of sulfite tolerance in *L. oenos*.

Cells of *L. oenos* X_2L cultured in ethanol 8% (v/v) secreted two proteases (I and II) which could affect the quality of the wine [35,123]. The proteolytic system of *L. oenos* is important for two reasons. First, these exoenzymes play a nutritional role in making peptides and amino acids (eg, arginine) available for cell growth. Second, they may cause turbidity affecting the stability of wine [93,125]. The effect of additional arginine on the production of biogenic amine needs to be further elucidated.

End products and sensory analysis

Beside L-malic acid, other substrates are metabolized by *L. oenos* during MLF. Mixed-substrate fermentation not only stimulates cell growth but also reorients the metabolic pathways of *L. oenos* thereby modifying the sensory attributes of wine. The metabolism of citric acid has recently been investigated. *L. oenos* metabolizes citrate to acetate and oxalacetate [127]. The latter is then decarboxylated to pyruvate which is immediately converted to acetate, ethanol, lactate, diacetyl, acetoin [118] or 2,3-butanediol [119,130]. Wine yeast also contributes to the formation of these products. In particular, *L. oenos* mainly produced meso-2,3-butanediol and some D- and L-2,3-butanediol [63]. During glucose-citrate co-fermentation at pH 4.0 and 5.0, the commercial strain *L. oenos* GM generated higher

levels of acetate (compared to glucose only). Since an increase in volatile acids are a well known cause of spoilage in wine, the use of citric acid should be considered with caution [62]. Volatile acids could also increase as a result of a slow/stuck alcoholic fermentation due to antagonistic activity between *L. oenos* and wine yeasts [64]. Little is known about the role of aeration in MLF. Oxidation conditions also influence the amount of end products produced from sugar metabolism (eg, of glucose, fructose and arabinose). In particular, under anaerobic conditions (CO_2 and N_2 atmosphere), acetic acid was accumulated by the metabolism of *L. oenos* GM, especially when fructose and malate were metabolized concurrently [36].

The term 'complexity' is often used to describe the contribution of microorganisms, during processing and aging, on the flavor of the wine. The primary objective of MLF induction in Chardonnary is to increase the wine complexity. Many acids, alcohols, esters and carbonyl compounds have been associated with MLF [5,143]. The contribution of individual compounds to the sensory effect of MLF has not yet been established [58]. However, acetic acid, diacetyl, acetoin and 2,3-butanediol are among the most important substances from an analytical point of view [62]. Diacetyl, a compound with a buttery or nutty flavor [81], has a threshold value varying from 0.2 to 2.8 mg L^{-1} for Chardonnay and Cabernet Sauvignon, respectively [94]. The utilization of diacetyl by *L. oenos* has been demonstrated [96]. This compound is reduced to acetoin and 2,3-butanediol, which usually have no influence on wine aroma [106]. Starter cultures differ in the amount of diacetyl produced/consumed. The final diacetyl concentration of Cabernet Sauvignon, Pinot Noir and Chardonnay wines was significantly affected by the strain of *L. oenos* used [97]. The concentration of diacetyl obtained during MLF was dependent on the oxygen concentration and the redox potential of the wine. The initial citric acid content and the SO_2 concentration influenced the diacetyl concentration to a lesser extent. The diacetyl-SO_2 complex may hydrolyze during storage of the wine [107]. If the amount of diacetyl is too high after completion of MLF, its reduction to acetoin could be achieved by leaving the bacteria in contact with the wine, or by a second inoculation of fresh yeast [61]. The concentration of diacetyl in 41 Chardonnay wines ranged between 0.005 and 1.7 mg L^{-1} [95]. Despite a significantly higher diacetyl concentration measured in wine which underwent MLF, the contribution of this compound to the flavour of Chardonnay was independent of MLF, indicating that diacetyl is not the only important flavour component.

There are further contradictory results on the effect of MLF on wine flavour [62]. Organoleptic changes seem to be strain-specific. Wines inoculated with different strains of *L. oenos* were discriminated by sensorial analysis. However, these differences were neither particularly significant nor reproducible [91]. MLF produced Chardonnay and Pinot Noir wines with significant lower acidity levels compared to control wines (without MLF). Interestingly, when these wines were adjusted to similar malic and lactic acid content, the acidity of each wine was still deemed to be different [76]. Other authors [7,42,122] reported only slight sensory differences between Chardonnay wines obtained with and without MLF conducted with different strains of *L. oenos*. On the other hand, sensory analysis indicated a significant difference in aroma of MLF wines when compared to control wines without MLF [5]. After MLF, acetic acid and isobutanol increased while the content of propionic acid and isobutyl acetate decreased. According to Henick-Kling and Acree [61] the malolactic starter cultures modify the aroma of wine to different extents. Some cultures may reduce the vegetative aromas more then others, leading to a more pronounced fruity aroma. Selected strains of malolactic bacteria may also release fruity aroma compounds (eg, damascenone). Sauvageot and Vivier [131] reported that MLF increased the hazelnut, fresh bread, and dried fruit aromas of Chardonnay wines, whereas Pinot Noir wines partially lost their berry notes in favour of animal and vegetable perceptions. Again, the aroma of Pinot Noir wines, characterized by 33 aroma descriptors, varied significantly with strain [100]. Rosi *et al* [126] reported a lack of buttery aroma in Chardonnay wine fermented with five strains of *L. oenos* (EQ 54, ED 77, E 355, E 366 and EQ 05). They found these strains unable to metabolize citric acid with the final concentration of diacetyl in the 0.03–0.05 mg L^{-1} range. Strains E 366 and EQ 05 produced a higher quantity of ethyl esters of short chain fatty acids, isoamyl acetate and acetic acid, which characterized the wine bouquet as a 'flower' type. On the contrary, strains EQ 54, ED 77 and E 355 lead to higher alcohol and acetaldehyde content, and a wine bouquet classified as 'fruity'. Grossmann and Heinemeyer [49] found that only small-scale MLF fermenters (400 liter) produced a Muller–Thurgau wine which was preferred by panel tasters to the corresponding deacidified wines. Not only the size but also the type of the fermentation tank (eg, wood *vs* steel) is thought to play an important role in MLF [148].

Beside the production of chemicals which improve the quality of wine, the formation of undesirable compounds should be also considered. Ethyl carbamate (urethane) and histamine are among the main health hazard compounds in wine [75,116]. Biogenic amines are generated by enzymatic decarboxylation of amino acids [17,132,135]. Moreover, arginine metabolism by *L. oenos* leads to the formation of urea, an ethyl carbamate precursor [87]. In fact, ethyl carbamate in wine is produced by the reaction between ethanol and urea [109]. The relation between MLF in wine and ethyl carbamate formation is subject to controversy [137,138].

Recently, Liu *et al* [83] found a correlation between arginine degradation and ethyl carbamate production during MLF caused by *L. oenos* in laboratory-vinified wine. The formation of amines is affected by several factors including the growth of microorganisms with specific decarboxylase enzymes and the availability of amino acids [85]. Liu *et al* [86] demonstrated degradation of arginine by *L. oenos* via the arginine deiminase pathway. *L. oenos* DSM 20252 utilized only the supplied tyrosine for cell growth and production of tyramine; other amino acids (histidine, lysine and ornithine) were not metabolized by this strain [13]. Many strains of *L. oenos* hydrolyzed arginine producing citrulline (molar ratios ranging from 0.02 to 0.33), ornithine and ammonia according to the arginine deiminase pathway [48]. *L. oenos* 9204 produced histamine from histidine via the enzyme histidine decarboxylase (HDC) [124]. In a syn-

thetic medium, this strain produced large amounts of histamine, especially when cultured in the absence of glucose and malic acid. Therefore, the amine production seemed not to depend on the concomitant degradation of malic acid. The histamine level increased at low pH and ethanol concentration and in the presence of yeast lees which released histidine in the medium [90]. According to Henschke [62], Switzerland has recently set a legal limit of 10 mg L^{-1} histamine in wine. Higher levels of putrescine, histamine, methylamine and tyramine were found after MLF. In particular, lees and marcs can contain high levels of these biogenic amines [6]. Soleas *et al* [134] extensively studied the content of nine biogenic amines in 73 wines from Ontario, Canada. They found higher amine contents in Pinot Noir and Chardonnay, putrescine and histamine being the most concentrated (up to 13 and 11 mg L^{-1}, respectively, in Pinot Noir). There was no correlation between length of skin contact and concentration of any biogenic amine measured. Gloria *et al* [45] found that putrescine, followed by histamine and cadaverine, were the most prevalent amines in 59 samples of Pinot Noir and Cabernet Sauvignon produced in Oregon, USA. The addition of lysozyme (500 mg L^{-1}) inhibited MLF and reduced the level of histamine, tyramine and putrescine four-fold compared to the control [43]. All the histidine decarboxylating bacteria (HDC^+) found in almost half of the 118 wines tested belonged to the *L. oenos* species. Histidine decarboxylase activity was still detected in the absence of a viable cell population. Therefore, the autolysis of this histamine-producing bacterium could also generate high levels of histamine during wine aging [17].

Conclusions

In conclusion, a good understanding of MLF is important in the manufacture of wine and offers a great potential for improving the quality of wine from cool growing regions displaying high acid content. In common practice, the native malolactic bacteria of grapes accomplish this fermentation in wine. Recently, freeze-dried starter cultures have become available to initiate this process. So far, the genetic engineering of *S. cerevisiae* to achieve MLF of wine was only partially successful, due to difficulty in expressing the malolactic gene in the host cell. The interactions among bacteria, yeasts and environmental conditions are complex and still not fully understood. Finally, the importance of methodology and training is central to sensorial analysis which provides a powerful tool for product analysis and development.

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