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Evaluating the influence of stress parameters on *Oenococcus oeni* and the subsequent volatile aroma composition of white wine

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Contents

List of abbreviations and symbols

AF	Alcoholic fermentation
AKB	α-Ketobutyrate
ATP	Adenosine triphosphate
CGL	Cystathionine-γ-lyase
DMDS	Dimethyl disulphide
DMTS	Dimethyl trisulphide
DNA	Desoxyribonucleic acid
CFU	Colony forming units
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
GC-PFPD	Gas chromatography –pulsed flame photometric detection
kDA	Kilodaltons
K_m	Michaelis constant
LAB	Lactic acid bacteria
MLF	Malolactic fermentation
MeSH	Methyl mercaptan
NAD	Nicotinamide adenine dinucleotide
PLP	Pyridoxal-5'-phosphate
rpm	Revolutions per minute
S	Substrate
TA	Total acidity
V_{max}	Maximum velocity
v/v	Volume by volume
VSC	Volatile sulphur compounds

1 Introduction

The production of high quality and well-balanced wines requires a judicious balance between the acid, the sugar and the volatile aroma composition. Several factors affect the final wine style such as the grape quality and phytosanitary status, the cultivar and the microorganisms involved during the vinification process. L-Malic and tartaric acids are the most prominent organic acids in wine and play an important role in the vinification process, including the organoleptic quality and the microbial, physical and biochemical wine stability (Volschenk et al. 2006). Malolactic fermentation (MLF) induced by lactic acid bacteria (LAB), a secondary fermentation for naturally reducing wine acidity, efficiently decreases the acidic taste of wine, improves the microbial stability and contributes to the flavour profile. However, the phenomenon of delayed or sluggish MLF often causes an interruption of the vinification process and still little is known about the sensorial contribution by LAB.

1.1 Lactic acid bacteria associated with wine

The term LAB refers mainly to the characteristic feature of the basal metabolism of these bacteria, the fermentation of hexose sugars primarily yielding lactic acid (Makarova and Koonin 2007). LAB are Gram-positive, anaerobic to aerotolerant, non-sporulating, acid tolerant bacteria and include both homofermenters and heterofermenters (Mayo et al. 2008). The homofermenters primarily produce lactic acid, while heterofermenters yield a variety of fermentation by-products, including lactic acid, acetic acid, ethanol, carbon dioxide and formic acid (Kleerebezem and Hugenholtz 2003).

The primary source of their metabolic energy is supplied in the form of ATP by substrate level phosphorylation (Kandler 1983; Konings 1985). LAB are highly demanding in terms of the nutritional composition of their growth media. Usually, in addition to carbon and energy sources, various amino acids, vitamins, nucleic acids and mineral components are required (Konings 2002).

The bacteria associated with spontaneous MLF in grape wine belong to different genera of LAB. They are present in all grape musts and wines (Lafon-Lafourcade et al. 1983).

Four genera were identified as the principal organisms involved in the MLF, namely: *Lactobacillus* (*Lb.*); *Leuconostoc* (*Lc.*); *Oenococcus* (*O.*) and *Pediococcus* (*P.*) (Lonvaud-Funel 1999). These genera have the ability to tolerate low pH, high ethanol concentration and to grow in wine.

In particular, *O. oeni* has especially the ability to adapt well to high ethanol concentrations (up to 15 % v/v), low pH (as low as 2.9) and limited nutrient conditions (Van Vuuren and Dicks 1993). These characteristics enable *O. oeni* to out-compete other potential MLF bacteria during the later stages of vinification and thus dominate in wine after alcoholic fermentation (AF), until the end of MLF (Bartowsky 2005). For these reasons and for it's least association with off-flavours or other undesirable metabolites, *Oenococcus* starter cultures are most widely used for winemaking (Mills et al. 2005).

1.2 Climate change-associated effects on LAB

A variety of factors affect the growth of LAB or their metabolic properties and consequently the timely completion of MLF. These comprise: ethanol content (> 13 % v/v), pH (< 3.2), SO_2 (> 10 mg/L free SO_2, more inhibitory at low pH); temperature (< 18 °C) (Henick-Kling 1993; Ribéreau-Gayon et al. 2006), and yeast metabolites (Alexandre et al. 2004; Lerm et al. 2010; Nehme et al. 2010). Among the most important climate change related effects are increased grape sugar concentrations that lead to high wine alcohol levels and lower acidities (Mira de Orduña 2010). MLF may equally be affected by high ethanol concentrations and cause stuck or sluggish fermentation which in turn compromises the vinification efficiency and wine quality by delaying ageing and stabilisation processes (Lonvaud-Funel 1999). A combination of inhibiting factors may result not only in difficult MLF in hot climate regions in the future, but also in cool climate regions where moderately raised ethanol levels may lead to inhibition in conjunction with low pH values (Mira de Orduña 2010).

1.3 Malolactic activity

MLF in wine is by definition the enzymatic conversion of L-malic acid (dicarboxylic acid) to L-lactic acid (monocarboxylic acid) and carbon dioxide (Henick-Kling 1993), a

secondary process which either follows AF of wine or occurs simultaneously. This decarboxylation reaction is catalysed by the malolactic enzyme (L-malate: NAD^+ carboxylase) (IUC number 1.1.1.38) in the presence of NAD^+ and Mn^{2+} (Kunkee 1991). The malolactic enzyme of *O. oeni* has been genetically characterised by Labarre et al. (1996). The *mle* locus of *O. oeni* consists of three genes: gene *mleA* that encodes the malolactic enzyme, gene *mleP* which encodes the malate-permease and the *mleR* gene, which encodes the regulator that activates transcription of the malolactic operon.

1.3.1 Bioenergetical aspects

O. oeni is well known for its ability to conduct MLF at more acidic pH values. Under these acid conditions it maintains a rather constant internal pH of 5.8-6.3 (Salema et al. 1994). The free energy of the decarboxylation reaction is conserved by a chemiosmotic mechanism (Salema et al. 1996b) which depends on an electrogenic malate transport (Konings 2002), thereby generating a membrane potential (inside negative) (Salema et al. 1994). For each negatively charged mono-protonated malate ($Hmalate^-$) molecule that enters the cell and is decarboxylated, one molecule of lactate leaves the cell including one proton (H^+), which is equivalent to the translocation of one H^+ to the external environment (Figure 1). This decarboxylation therefore results in the alkalinisation of the cytoplasm and the generation of a pH gradient (ΔpH) (Konings 2002). The resultant increase in proton motive force can be used by a membrane ATPase to produce ATP at low pH (Cox and Henick-Kling 1989), as well as for the uptake of nutrients (Unden and Zaunmüller 2009) and to keep suitable internal conditions for enzyme activity and growth under conditions of acidic pH as in wine (Salema et al. 1996b).

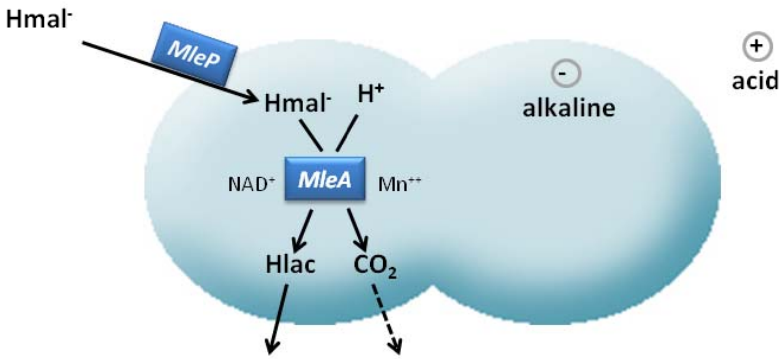

Figure 1. Metabolic energy conservation by malate transport and proton consuming decarboxylation in *O. oeni*. Uptake of mono-anionic malate ($Hmal^-$), proton consumption by malate decarboxylation and passive efflux of undissociated lactate (Hlac). Important enzyme and carrier: *MleP* malate carrier; *MleA* malolactic enzyme (adapted after Konings 2002).

1.3.2 The impact of ethanol and pH

Various stress factors in wine such as low pH and high ethanol concentration, have a negative impact on LAB (Spano and Massa 2006). Biological membranes are the primary target of stress injury, and it is believed that membrane physical properties and lipid composition are the main factors involved in environmental stress. Ethanol and low pH strongly affect the physical and chemical properties of the cytoplasmic membrane of LAB (Cotter and Hill 2003; da Silveira et al. 2003, 2004) and disrupt the capacity for pH homeostasis (Jordan et al. 1999; Barker and Park 2001). The malolactic activity of the cells is strictly dependent on the integrity of the bacterial membrane. Due to the pH optimum (~ pH 5.8) of the malolactic enzyme, the requirement of Mn^{2+} and NAD^+, as well as other possible inhibiting wine components, the protein needs to be protected from the medium by the cell membrane (Lonvaud-Funel 1999).

The amount of ethanol tolerated, is highly strain dependent. However temperature, pH and nitrogen status of the medium also play a role (Henick-Kling 1993). The effect of ethanol on the cell envelope has been broadly investigated in *O. oeni*. The ethanol toxicity is generally attributed to the preferential partitioning of ethanol in the hydrophobic environment of lipid bilayers, resulting in a disruption of the membrane structure that negatively affects many membrane-associated processes (da Silveira et al. 2002, 2003, 2004).

It was shown, that the membrane composition of *O. oeni* is strictly dependent on ethanol concentration and the cell physiological state (da Silveira et al. 2003, 2004). The

fluidity of the cytoplasmic membrane in *O. oeni* cells instantaneously increased with the addition of ethanol, in a concentration-dependent manner (da Silveira et al. 2004; Chu-Ky et al. 2005). It was observed, that during cultivation in ethanol, the cells modify the composition of fatty acids in the membrane by firstly increasing the proportion of cyclic fatty acids (Teixeira et al. 2002) and secondly by increasing the membrane protein / phospholipid ratio, to limit the effect of ethanol on lipids (da Silveira et al. 2003).

Teixeira et al. (2002) demonstrated that *O. oeni* maintained a high level of phospholipid biosynthesis via the relative increased biosynthesis of phosphoethanolamine and sphingomyelin in the presence of ethanol. In addition, ethanol induced an increase in the membrane lactobacillic acid percentage, which appeared to be a factor that provides protection against the toxic effect of ethanol, balancing the increase of membrane fluidity normally attributed to ethanol (Teixeira et al. 2002). Furthermore, it has been reported that ethanol stressed cells of *O. oeni*, adjust their membrane permeability during ethanol adaption by decreasing fluidity at the lipid water interface (da Silveira et al. 2004). A combined ethanol and acid shock has been shown to induce strong membrane rigidification, indicating a highly disorganised state of the cell membrane (Chu-Ky et al. 2005).

Research has shown that membrane disordering resulting from ethanol exposure leads to leakage of intracellular compounds, including enzymatic co-factors (NAD / NADH) and ions essential for cell growth and fermentation, as well as dissipation of the electrochemical gradient across the cytoplasmic membrane (Spano and Massa 2006) resulting in less effective energy transduction (Sikkema et al. 1995). An influx of protons can then occur which will influence cell processes dependent on the pH gradient such as ATP synthesis, transportation of amino acids and L-malate (Guzzo and Desroche 2009).

Ethanol has therefore a crucial impact on the physiology of cells, because its presence generates important modifications that are the basis for adaption of the cells to this stress (Guzzo and Desroche 2009).

Generally, the pH optimum for LAB is close to neutrality (Hutkins and Nannen 1993). Some families of LAB such as *Lactobacillus* and *Oenococcus* show more acidophilic behaviour. During the vinification process, the average pH is between 3.0 and 3.8; at

pH values less than 3.0, bacterial growth is very difficult or impossible dependent on other physical and chemical factors (Lonvaud-Funel 1995).

Weak acids have potent protonophor activity, because the undissociated form of weak acids pass freely through the cell membrane. In case of an external pH lower than the cytoplasmic pH, the weak acids dissociate, releasing a proton and leading to acidification of the cytoplasm (Cotter and Hill 2003), thus inhibiting intracellular enzymes and proton motive force dependent transport systems (Henick-Kling 1993).

The activation of MLF to generate a proton motive force so as to maintain the intracellular pH (Salema et al. 1996a; 1996b), has been associated with a possible acidic stress response (Tourdot-Marechal et al. 1999; Guzzo et al. 2000). This homeostasis of the internal pH is essential for the growth and survival of the cells, as many metabolic pathway enzymes function optimally around neutral pH and their activities decrease at lower or higher pH values (Konings 2002). In environments with acid pH values, additional proton removing processes, such as MLF, are therefore needed to maintain the internal pH. These activities ultimately result also in an increase of the external pH. An additional function of MLF thus lies in preventing acid-killing of the cells by the opposing effect of the acidification of the external pH (Konings 2002).

It was observed that sugar utilisation and growth of *O. oeni* are inhibited by low pH, whereas the rate of L-malic acid degradation is highest at low pH (<4.5) (Henick-Kling 1993). Despite the fact that *O. oeni* is not able to grow with L-malic acid as the sole carbon source, research has indicated that intact cells generate more ATP when grown at low pH in the presence of L-malic acid (Cox and Henick-Kling 1989; Cox and Henick-Kling 1990).

The pH is therefore an essential factor in wine and has several consequences regarding the success of MLF: selection of the best adapted strains; impact on growth rate and yield; influence on the malolactic activity; and effect on the substrates transformed.

1.4 Timing of bacterial inoculation

There are different LAB inoculation possibilities, such as simultaneous inoculation of yeasts and LAB for alcoholic and malolactic fermentation (co-inoculation), inoculation of LAB during AF and inoculation after the completion of AF (sequential inoculation) (Davis et al. 1985). Simultaneous inoculation can be an efficient alternative to overcome the potential inhibition of LAB, due to high ethanol concentrations and reduced nutrient content (Jussier et al. 2006; Zapparoli et al. 2009). Hence, a more successful induction of MLF due to a gradual adaption of bacteria to increasing alcohol concentrations and due to the benefit from higher nutrient availability present in the must, compared to the condition at the end of AF (Rosi et al. 2003). Likewise, simultaneous inoculation of musts / wines with high acidity but still low levels of ethanol and higher nutrient concentration may help to avoid potential MLF problems. Furthermore it would be beneficial regarding technical aspects. Wines after successful co-inoculation would be immediately ready for downstream treatments, such as racking, fining and sulphur dioxide addition, thus increasing microbiological stability and processing efficiency (Jussier et al. 2006).

1.5 Yeast-bacteria interactions

Independently of the LAB inoculation time, the development of undesirable / antagonistic yeast–bacteria-interactions should be considered (Henick-Kling 1995). Alexandre et al. (2004) reviewed the interactions between *Saccharomyces cerevisiae* and *O. oeni* in wine and reported that yeasts can oppose or stimulate MLF. Therefore, successful MLF also will strongly depend on the careful selection of suitable yeast-bacterium combinations (Alexandre et al. 2004; Jussier et al. 2006).

Alexandre et al. (2004) proposed that the degree and complexity of these interactions are dependent upon three factors, including the yeast/bacteria strain combination, the uptake and release of nutrients by yeast, and the ability of the yeast to produce metabolites that are either stimulatory or toxic to the bacterium. The yeast metabolites, summarised in Table 1, comprise medium chain fatty acids (hexanoic, octanoic, decanoic and dodecanoic acid), SO_2, ethanol as well as metabolites of protein nature and their production is affected by yeast strain, medium composition (e.g. degree of clarification of

grape must) or winemaking practices (e.g. skin contact, ageing on yeast lees). Both, bacterial growth rate and malolactic activity, are influenced by these metabolites, depending on their concentration but also on the pH of the medium (Alexandre et al. 2004). However, inhibition of these toxics is not only dependent on the yeast strain, but also on the bacterial strain, as ethanol or sulphite tolerance for instance, are very different among the bacteria species and between strains of the same species (Davis et al. 1988). In addition, pH will indirectly influence sulphite and ethanol tolerance, resulting in synergistic inhibition by low pH, SO_2 and ethanol (Britz and Tracey 1990; Guerzoni et al. 1995).

Possible stimulating effects of yeast on LAB growth and MLF may result from protease activities, macromolecule (e.g. mannoproteins) production (Guilloux-Benatier et al. 1995) and autolytic capacity (Patynowski et al. 2002).

Table 1. Yeast metabolites with inhibiting effect on LAB (adapted from Lerm et al. 2010).

Yeast metabolite	**Influence on LAB and/or MLF**	**Reference**
Ethanol	Affects growth rate and length of lag phase rather than malolactic activity. Acts synergistically with low pH.	Henick-Kling (1993)
SO_2	Inhibitory effect on growth and malolactic activity. Acts synergistically with low pH.	Wibowo et al. (1985), Henick-Kling and Park (1994)
Medium chain fatty acids	Affect growth and reduce ability to metabolise malic acid. Combination of fatty acids causes greater inhibition than individual compounds. Act synergistically with ethanol.	Lonvaud-Funel 1988, Edwards et al. 1990, Alexandre et al. (2004)
Peptides and proteins	Affect growth and reduce malolactic activity.	Dick et al. (1992), Mendoza et al. (2010), Nehme et al. 2010

1.6 Beneficial effects of MLF in wine

In wine the transformation of malic acid causes a dual effect, the first being the deacidification by an increase of the initial pH (0.1-0.2 units) and the second being a softening of the mouthfeel. The acidic and astringent flavour of the malic acid is replaced by the smoother aroma of the lactic acid (Lonvaud-Funel 1999; Bartowsky 2005). Red wine

production in both cold and warm climate regions usually involves MLF, naturally or induced. Spontaneous MLF occurs less frequently in white wines due to an average lower pH of most white cultivars (Volschenk et al. 2006). The rate of malate decarboxylation in wine is directly linked to the cell number of LAB; to specific malolactic activity (Henick-Kling 1993); to the physiological state of the bacterial cells (Versari et al. 1999) and to the physico-chemical properties of the wine. A significant rate of MLF is not usually observed until the cell density exceeds 10^6 CFU/mL (Lonvaud-Funel 1995).

Depending on the initial pH of the must, the degradation of L-malic acid via MLF can be either beneficial or negative to wine quality. In low pH wines, generally found in cool climate regions, a decrease is favourable for the production of acid-balanced wines (Henick-Kling 1995; Lonvaud-Funel 1999). Whereas, in warm climate regions, flavour changes from MLF are of more importance than the acid reduction (Henick-Kling and Acree 1998).

1.7 Impact of MLF on wine aroma composition

Although the primary role of LAB is the transformation of L-malic acid, they are also involved in the production of other minor, but important, aroma active metabolites (Figure 2). Some of these compounds are found in wine at or above their sensory threshold and a variety of descriptors, positive and negative, have been listed which include buttery, nutty, vanilla (Bartowsky et al. 2002), fruity, reduced vegetative aromas (Henick-Kling 1993), acetic and rancid yoghurt amongst others (Palacois 2006).

Recent research has focused on the organoleptic changes in wine following MLF and various studies have shown that numerous individual flavour-active compounds produced by bacteria contribute to wine aroma changes during MLF (Davis et al. 1985; Laurent et al. 1994; Henick-Kling 1995; Lonvaud-Funel et al. 2002). Important aroma compounds responsible for MLF flavour characteristics were recently reviewed in detail by Lerm et al. (2010). MLF is generally associated with increased concentrations of carbonyl compounds (e.g. diacetyl) (Nielsen and Richelieu 1999), ethyl esters, including lactic acid ethylester, acetic acid ethylester, hexanoic acid ethylester or octanoic acid ethylester (de Revel et al. 1999; Delaquis et al. 2000; Liu 2002, Boido et al. 2009), as well as higher levels of succinic acid diethylester, acetic acid phenylethylester or acetic

acid 3-methylbutylester (Maicas et al. 1999). Moreover, the release of glycosidically bound flavour compounds, such as monoterpenes and C_{13}-norisoprenoids, has been observed in wines after MLF (D'Incecco et al. 2004; Hernandez-Orte et al. 2009).

Various factors have to be considered when investigating the impact of LAB and MLF on the wine volatile aroma composition. The changes of aroma compounds can be affected by the bacterial strain chosen (Versari et al. 1999), the timing of LAB inoculation (Bartowsky et al. 2008), as well as the grape cultivar or winemaking practices (e.g. barrel or tank fermentation) (Henick-Kling and Acree 1998; Bartowsky et al. 2009). However, to date, few of these components and the mechanisms involved in their production have been identified. Swiegers et al. (2005) listed the possible pathways by which LAB are able to produce volatile compounds by e.g. metabolising grape components (e.g. sugars and nitrogen containing compounds) or modifying of yeast derived secondary metabolites, such as esters or higher alcohols (Figure 2). Different studies investigated the specific biochemical activities of LAB involved in the formation of volatile aroma compounds, mostly carbonyl compounds (Bartowsky and Henschke 2004; Saguir et al. 2009), esters (Matthews et al. 2007; Sumby et al. 2009) or monoterpenes (Bodio et al. 2002; Ugliano et al. 2003; Barbagallo et al. 2004).

The following section will focus on the main aroma compounds associated with MLF, as well as on some key factors that influence their formation.

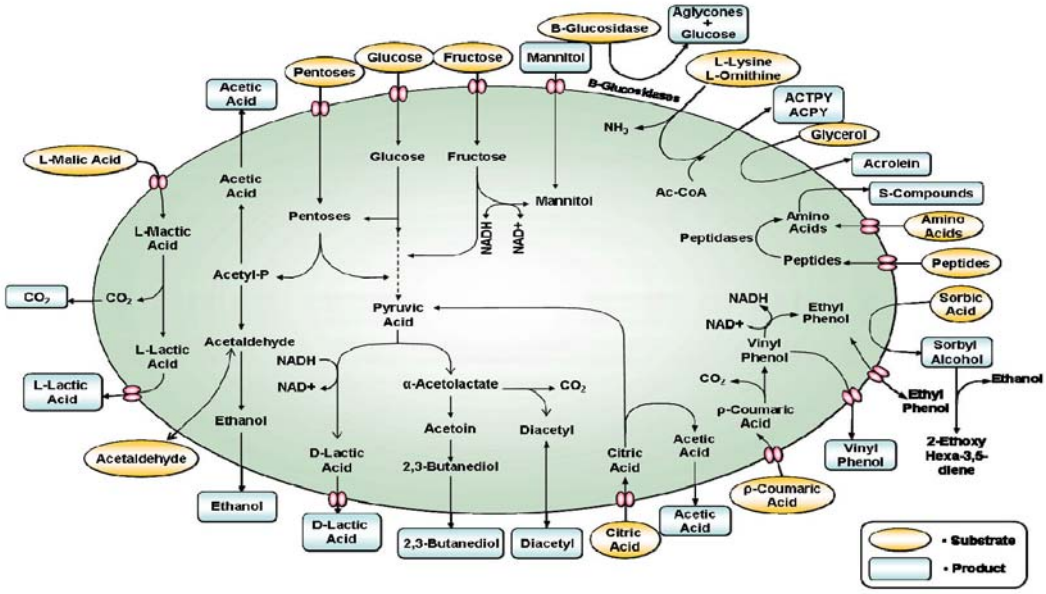

Figure 2 A schematic representation of the biosynthesis and modulation of flavour-active compounds by LAB (reprinted with permission from Swiegers et al. 2005).

1.7.1 Carbonyl compounds

Among the sensorial changes which originate from LAB during MLF, the carbonyl compounds diacetyl, acetoin and 2,3-butandiol are considered to be one of the most important flavours. The buttery – diacetyl-attribute, reviewed by Bartowsky and Henschke (2004), has in moderate concentrations (~ 1-4 mg/L) a positive effect on the wine bouquet, while at higher concentrations (> 5-7 mg/L) it becomes a defect.

The formation and degradation of diacetyl is directly related to the growth of LAB and the metabolism of sugar, malic acid and citric acid (Swiegers et al. 2005). It is formed as an intermediate metabolite in the reductive decarboxylation of pyruvic acid to 2,3-butanediol (Ramos et al. 1995), and diacetyl is the product resulting from the chemical oxidative decarboxylation of α-acetolactate (Bartowsky and Henschke 2004). Pyruvic acid is derived from the metabolism of sugar and citric acid, and the formation of 2,3-butanediol might contribute to the redox balance of cellular metabolism (Bartowsky and Henschke 2004). Due to the fact that diacetyl is chemically unstable, it is further reduced to acetoin, which in turn can be reduced to 2,3-butanediol (Bartowsky et al. 2002). Maicas et al. (1999) detected decreased concentrations of diacetyl after MLF,

but increased concentrations of 2,3-butanediol as a result of enzymatic reduction of diacetyl by LAB.

A variety of factors, including some that the winemaker can control, influence the concentration of diacetyl in wine, such as oxygen exposure, fermentation temperature, SO_2 levels, duration of MLF, as well as bacterial strain (Bartowsky and Henschke 2004). An important role plays also the rate of MLF. Lower levels of diacetyl and acetoin are produced at a higher MLF rate. By selecting a bacteria strain that possess the ability to produce higher concentrations of diacetyl, in conjunction with manipulating the temperature, SO2 content and lees contact, a winemaker is able to influence the diacetyl content according to the style of wine required (Lerm et al. 2010).

1.7.2 Esters

Esters are largely responsible for the fruity aroma of wine (Ebeler 2001). These are formed when alcohol and carboxylic acid functional groups react, and a water molecule is eliminated (Sumby et al. 2010). In wine, esters can be classified into two groups, those produced enzymatically and those formed by chemical esterification between alcohol and acids at low pH (Margalit 1997). Enzymatic ester synthesis by microorganisms in wine is catalysed by esterases, lipases and by alcohol acetyltransferases and has recently been reviewed by Sumby et al. (2010). The two main groups of fermentation-derived esters that have been associated with wine fruitiness are acetate esters and ethyl fatty acid esters (Ugliano and Henschke 2009). The acetate esters are comprised of an acid group (acetate) and an alcohol group which is either ethanol or a complex alcohol derived from amino acid metabolism (Saerens et al. 2008). Ethyl esters comprise of an alcohol group (ethanol) and an acid group (medium-chain fatty acid) (Saerens et al. 2008).

Even though the esterase activity of *O. oeni* is not well documented, it is clear that MLF and wine LAB have the ability to alter the ester content of wine. The majority of *O. oeni* and *Lactobacillus* strains evaluated by Davis et al. (1988) showed esterase activity and similarly, all strains screened by Matthews et al. (2006) could hydrolyse esters. Moreover, Matthews et al. (2007) observed that esterases showed greater activity towards short-chained esters (C_2 to C_8) in comparison to long chained esters (C_{10} to C_{18}).

As mentioned before, increases of ester concentrations in wines following MLF, including acetic acid ethylester, lactic acid ethylester, succinic acid ethylester, as well as decreases in some esters have been documented (Laurent et al. 1994; Maicas et al. 1999; Ugliano and Moio 2005; Bartowsky et al. 2008). Indeed, Bartowsky et al. (2008; 2009) observed a consistent increase of mostly ethyl esters and a decrease of acetate esters in wines following MLF.

Lactic acid ethylester and succinic acid diethylester are important esters that typically play a role in MLF and most of the time show quantitatively the largest concentration increase (Maicas et al. 1999; Herjavec et al. 2001; Ugliano and Moio 2005). Lactic acid ethylester is associated with an increased mouthfeel of the wines, as well as with its contribution to fruity, buttery and creamy aromas (Ugliano and Moio 2005). It is the esterification product of lactate, produced by LAB during MLF, and ethanol present as a result of AF (Maicas et al. 1999) and its accumulation is dependent on malic acid metabolism (Ugliano and Moio 2005). Succinic acid diethylester arises from esterification of succinic acid, a byproduct of microbial α-ketoglutarate metabolism (Radler 1986), hence its increase with MLF, together with other related esters such as 4-hydroxybutanoic acid ethyl ester (Ugliano and Moio 2005).

1.7.3 Monoterpenes

Monoterpenes (e.g. linalool, α-terpineol) are important aroma active compounds, contributing floral, fruity and citrus attributes (Strauss et al. 1986). The release of these grape-derived, non-volatile, flavourless and glycosidically-bound aroma compounds can be achieved by the action of glycosidase enzymes or via an acid-catalysed process (Ugliano 2009). Acid hydrolysis is however fairly slow under typical vinification conditions, and is mainly regarded as a pathway for the formation of the wine ageing bouquet (Sefton 1998). On the other hand, the action of glycosidase enzymes can rapidly hydrolyse the aroma precursors and release the bound volatile compounds (Günata et al. 1993). Recent studies reported significant β -glycosidase activities in different *O. oeni* strains in model systems (Grimaldi et al. 2000; Ugliano et al. 2003; D'Incecco et al. 2004; Grimaldi et al. 2005; Hernandez-Orte et al. 2009) and during red wine production (Bodio et al. 2002; Ugliano and Moio 2006). These results suggest that the LAB of wine have the potential to hydrolyse glycoconjugates that positively affect the wine aroma. However, the latter studies observed that the degree of the release of glycosidically

bound aroma compounds tended to be strain- and grape cultivar-dependent, and was also influenced by the chemical composition of the medium.

1.7.4 Volatile sulphur compounds

Few studies are being undertaken into the specific biochemical activities linked to the production of other interesting flavour active compounds. At present, the metabolism of sulphur-containing amino acids by wine LAB is not well known. In contrast, this metabolism by dairy LAB is thoroughly documented (Weimer et al. 1999; Seefeldt and Weimer 2000; van Kranenburg et al. 2002; Liu et al. 2008). First studies on these metabolic pathways in wine have demonstrated that *O. oeni* is able to produce, from methionine, different sulphonated components with an organoleptic effect, such as methanethiol, methyl disulphide (Rauhut et al. 2008a, 2008b), 3-(methylsulphanyl)propan-1-ol and 3-(methylsulfanyl) propionic acid (Pripis-Nicolau et al. 2004; Vallet et al. 2008). This latter compound is characterised by ‚chocolate' and ‚roast' aromas and significantly contributes to the aromatic complexity of red wines (Pripis-Nicolau et al. 2004), while the others are more likely to have 'cooked cabbage', 'onion' or 'cauliflower' odours (Mestres et al. 2000). Vallet et al. (2008) proposed a possible pathway by which these compounds are formed by *O. oeni* and suggested that 2-oxo-4-(methylthio)butyric acid plays a central role in volatile sulphur compound synthesis. However, no specific enzymes have been identified and characterised yet. The formation of volatile sulphur compounds (VSC) plays an important role in the complexity of wine aroma, because of their characteristic odours. Concentrations below or close to the threshold will add to complexity, while increased concentration will impart negative aromas to the wine (Landaud et al. 2008).

For a long time, the only role of LAB in winemaking was thought to be to degrade L-malic acid. Evidently, several g/L of L-malic acid are transformed while the other reactions merely involve a few mg/L or less of substrates. Chemical as well as sensorial analyses have shown that secondary metabolisms occur and positively or negatively affect the wine aroma. Yet, it is still not well known which species or strains grow at all, or at what time during the vinification process, and in the end which enzymatic activities they possess and what are the substances produced under certain stress conditions.

1.8 Objectives of the study

The process of MLF in wine and the impact of LAB on the wine aroma is only partially understood and difficult to predict.

The underlying objective of the work was to investigate the impact of partial and complete MLF on the volatile aroma composition of white wines (Riesling and Chardonnay).

The specific aims of this study were as follow:

- Evaluation of the impact of simulated cool and warm climate stress (pH and alcohol) on LAB performance and the volatile aroma composition of white wines;
- Assessment of the influence of different inoculation strategies of MLF on the organoleptic profile of wine and
- Identification of relevant enzymes in the sulphur metabolism of *O. oeni*.

The musts and wines used in this study were produced in two climatically different wine growing regions, namely Stellenbosch, South Africa and Geisenheim, Germany.

2 Publications

International peer reviewed scientific publications:

Knoll C, Fritsch S, Schnell S, Grossmann M., Rauhut D, du Toit M, 2011, "Influence of pH and ethanol on malolactic fermentation and volatile aroma compound composition in white wines", **LWT-Food Science & Technology,** in press, corrected proof: DOI: 10.1016/j.lwt.2011.05.009

Knoll C, Fritsch S, Schnell S, Grossmann M, Krieger-Weber S, du Toit M, Rauhut D, 2011, "Cool climate Riesling wines: Impact of different malolactic fermentation inoculation scenarios on wine aroma", **World Journal of Microbiology and Biotechnology** (submitted)

Knoll C, du Toit M, Schnell S, Rauhut D, Irmler S, 2011, "Cloning and characterisation of a cystathionine β/γ-lyase from two *Oenococcus oeni* oenological strains", **Applied Microbiology and Biotechnology**, 89, 1051–1060

2.1 Influence of pH and ethanol on malolactic fermentation and volatile aroma compound composition in white wines

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Influence of pH and ethanol on malolactic fermentation and volatile aroma compound composition in white wines

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ABSTRACT

The present study investigated the influences of pH and ethanol on malolactic fermentation (MLF) and the volatile aroma profile of the subsequent white wines from Riesling and Chardonnay inoculated with two different *Oenococcus oeni* strains. In all cases MLF was induced after completion of alcoholic fermentation (AF). Partial MLF occurred under low pH 3.2 and high alcohol (118.3 g/L) conditions. In the cases with complete MLF, the time required for each strain varied from 13 to 61 days and was dependent on bacterial culture, cultivar and wine parameter. Chemical properties of each wine were determined after AF, complete and partial MLF. The wines showed significant differences in total higher alcohols, esters and acids that are important for the sensory profile and quality of wine. This work demonstrated that the wine matrix as well as the pH and alcohol concentration affects MLF and the final volatile aroma profile. Results indicate that changes in volatile aroma composition are not necessarily related to complete MLF and that partial MLF already has distinct influences on the wine aroma profile of white wines.

1. Introduction

Malolactic fermentation (MLF) is a secondary fermentation in the vinification process and is characterised by the conversion of L-malic acid to L-lactic acid and carbon dioxide. It is conducted by lactic acid bacteria (LAB) and may occur spontaneously or be induced by the inoculation of commercial bacterial starter cultures (Lonvaud-Funel, Singh, & Stapleton, 2002). MLF influences three different, but linked, facets of wine quality: acidity, microbial stability and sensorial complexity of wine. These contributions made by MLF, can improve the wine quality and are desired, however, the same contributions may be regarded as undesirable under a different set of circumstances as found in cool – versus warm – climate wine regions (Volschenk, van Vuuren, & Viljoen-Bloom, 2006). The success of MLF is influenced by several oenological parameters, such as pH, temperature, alcohol content and SO_2 concentration (Henick-Kling, 1993). Low pH (3.0) and high alcohol concentration (up to 126.24 g/L) are two stress factors that when combined with other oenological parameters, influence the survival of LAB, and consequently MLF (Jackson & Lombard, 1993; Lonvaud-Funel, 1995) Wines from cool climate regions (e.g. northern Europe, New Zealand, Canada or northeast USA) are usually characterised by low pH and lower alcohol content. Whereas wines from warm climate regions (southern Europe, South Africa or Australia) are characterised by high alcohol levels due to high sugar concentrations and high pH levels (Volschenk et al., 2006). Of all species of LAB associated with wine, *Oenococcus oeni* is the most tolerant to the harsh environmental wine conditions and the most desired bacterial species to conduct MLF (Mills, Rawsthorne, Parker, Tamir, & Makarova, 2005). The metabolic activity, as well as the kinetics of MLF, will influence the sensory profile of the wine linked to the vinification techniques, the physical and chemical composition of the wine (Krieger-Weber, 2009). Swiegers, Bartowsky, Henschke, and Pretorius (2005) listed the possible pathways by which LAB are able to produce volatile compounds by e.g. metabolising grape components or modifying of yeast derived secondary metabolites or by the production of flavour-active compounds (Hernandez-Orte et al., 2009; Lonvaud-Funel, 1999; Maicas, Gil, Pardo, & Ferrer, 1999; de Revel, Martin, Pripis-Nicolau, Lonvaud-Funel, & Bertrand, 1999).

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Various descriptors, positive and negative, have been listed which include buttery, nutty, vanilla, fruity, vegetative, acetic and rancid yoghurt amongst others (Bartowsky, Costello, & Henschke, 2002; Henick-Kling, 1993; Jackson & Lombard, 1993; Liu, 2002; Palacois, 2006). Many of these alterations are strain dependent, however the vinification technique can also affect the final wine aroma profile.

Recent research proposed several mechanisms which enable *O. oeni* to withstand stress conditions which have been summarised by Spano and Massa (2006). (Zapparoli, Tosi, Azzolini, Vagnoli, & Krieger, 2009). Little is known about the influence of stress, such as pH and ethanol, on the volatile aroma compound production of LAB. Olguín, Bordons, and Reguant (2009) studied the impact of ethanol and pH on the gene expression of the citrate pathway in *O. oeni* and showed that the expression of citrate pathway genes was mainly affected by ethanol, while pH had a lower effect. Furthermore, the differences observed in gene expression were in correlation with the different content of end products such as diacetyl and acetic acid.

In order to evaluate the influence of pH and ethanol on MLF and the volatile aroma fraction of the white wines Riesling and Chardonnay, this study was conducted over two vintages in two climatically different winegrowing regions. The main objective of this work was to investigate the impact of partial and complete MLF on the volatile aroma composition. In all cases, MLF was done over a range of different pH values and alcohol levels and induced after completion of alcoholic fermentation (AF) by inoculation with two different *O. oeni* strains.

2. Materials and methods

2.1. Bacterial strains, medium and growth conditions

MRS-T agar (MRS agar containing 100 mL/L tomato juice, pH 5) was used for *O. oeni* strains R1105 and R1106 (Lallemand, Toulouse, France). The medium contained 50 mg/L Actistab (DSM Food Specalities Dairy Ingredients, Delft, The Netherlands) dissolved in distilled water which inhibited moulds and yeasts. Kanamycin sulphate (Roche Diagnostics, Mannheim, Germany) dissolved in sterile distilled water at 30 mg/L inhibited acetic acid bacteria. The bacterial population was determined weekly during MLF. Plates were incubated anaerobically at 30 °C for 7 days. Colony counts were carried out and reported as cfu/mL.

2.2. Micro-vinification

Two different micro-vinifications were conducted. Riesling grapes from Rheingau wine region (Germany) were harvested during the 2008 vintage. The Chardonnay grapes were harvested during the 2009 season and originated from Paarl wine region (South Africa).

Grapes were destemmed, crushed and 30 mg/L potassium metabisulphite were added. The must was settled over night at 15 °C, racked and analysed for sugar content, total acidity and pH (Table 1). The must was then inoculated with 30 g/hL of the commercial yeast starter culture Lalvin QA23 YSEO (Lallemand, Germany). The yeast was rehydrated according to the manufactures recommendations. Fermentations were carried out in stainless steel tanks at 20 °C in a temperature controlled room.

At the end of alcoholic fermentation, the wine was racked and then divided into 5 L glass bottles with S-shaped airlocks filled with water.

Before inoculation with the bacteria strains the pH of the wines was set to 3.2, 3.6 and 3.8 with 5 mol/LNaOH or 5 mol/L HCl. The alcohol was adjusted to 98.6 g/L and to 118.3 g/L with 99.8% ethanol p.a. (Roth, Karlsruhe, Germany). Two *O. oeni* strains (R1105, R1106) were used. The strains were inoculated at approximately 5×10^6 cfu/mL. The bacterial cultures were pre-cultured in 5 mL MRS-A broth (200 mL/L apple juice, pH 5.2) for 48 h–72 h at 30 °C. From this pre-culture 1.5 mL/100 mL was then inoculated into MRS broth containing D-glucose (20 g/L), D-fructose (40 g/L), DL-malic acid (4 g/L), Tween80 (1 g/L) and 31.56 g/L ethanol. The pH was adjusted to 4.6. After 48 h the pre-culture was centrifuged at 8000 rpm for 10 min, resuspended in water and inoculated into the wine. MLF was carried out at 20 °C in triplicate. To each wine sample, 50 mg/L of sulphite were added immediately upon completion of MLF. All samples were cold stabilised at 4 °C and bottled without prior addition of further fining agents or filtration.

Alcoholic fermentation (AF) was monitored by ethanol production and sugar depletion. MLF was monitored by malic acid degradation and lactic acid production. MLF was considered complete when malic acid was less than 0.1 g/L.

2.3. Analysis of must and wine for organic acids

Musts were analysed at the time of crushing and wine samples were collected during and after AF and MLF by Fourier Transform Infrared (FT-IR) spectroscopy and high performance liquid chromatography (HPLC). FT-IR analysis was performed according to Nieuwoudt, Prior, Pretorius, Manley, and Bauer (2004) using a Winescan FT120 equipped with a purpose built Michelson interferometer. HPLC analysis was performed according to Schneider, Gerbi, and Redoglia (1987) with following modifications: 5 µL of sample were injected into the Agilent Technologies 1100 series liquid chromatograph equipped with a multiwavelength detector (MWD) and analysed using an Allure® Organic Acid column (250 mm × 4.6 mm inside diameter) (Restek GmbH, Bad Homburg, Germany) with a Security Guard™ Cartridge C18 4 × 3 mm (Phenomenex, Aschaffenburg, Germany). The eluent was distilled water with 255.76 mg/L sulphuric acid and 3.95 g/L ethanol. The column was operated at 46 °C with an eluent flow rate at 0.6 mL/min. Eluting compounds were detected by UV absorbance at 210 nm. Enzymatic kits (Boehringer, Mannheim, Germany) were used for the analysis of SO_2-binding compounds according to the manufacturer's instructions.

Must components are reported as a single value without standard deviation. Wine analyses are reported as the means of three determinations (one for each trial carried out in triplicate).

Table 1
Analytical parameter of the musts and wines after alcoholic fermentation.

Sample	pH	Volatile acidity (g/L)	Total acid (g/L)	Malic acid (g/L)	Lactic acid (g/L)	Total sugar (g/L)	Ethanol (g/L)
Riesling must '08	3.2	0.13	9.8	4.9	nd	217	nd
Chardonnay must '09	3.3	0.18	5.4	4.3	nd	181	nd
Riesling wine '08	3.2	0.38	9.6	4.2	0.13	7.34	89.95
Chardonnay wine '09	3.3	0.29	4.9	4.2	0.12	2.43	86.79

nd not detected.

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2.4. Gas chromatography - mass spectrometry (GC–MS) analysis for volatile aroma compounds

GC–MS analysis was performed using a GC Hewlett Packard (HP) 5890 Series II (Agilent, Santa Clara, USA), coupled to a 5972 HP Mass Selective Dectector (Agilent). The GC was fitted with a cooled injection system (CIS 3) (Gerstel GmbH, Mülheim, Germany). Compounds were separated on a Varian VF-5MS column (Palo Alto, USA) with dimensions of 60 m × 0.32 mm × 1 μm. Analysis was done according to Rapp, Yavas, and Hastrich (1994), with the following modified conditions: injection was splitless (1 min) with the injector start temperature of 30 °C and then increased to 230 °C at 12 °C/min, and held for 4 min. The initial oven temperature was 40 °C, held for 5 min, then increased to 125 °C at 3 °C/min, further increased to 200 °C at 6 °C/min and held for 14.2 min. Helium was used as carrier gas at a constant flow rate (1 mL/min). The mass spectrometer was set to scan mode, covering a mass-to-charge ratio range (m/z) from 35 to 250 atomic mass units (amu). The temperature of the MS was set to 180 °C, respectively.

2.5. GC–MS solid-phase microextraction (SPME) analysis for carbonyl compounds

Analysis of diacetyl and acetoin was done according to Hayasaka and Bartowsky (1999), modified by Malherbe (2011) with following conditions: The GC–MS analysis was carried out using a gas chromatograph (Agilent Technologies, model 6890N, Network GC system, USA) combined with a mass selective detector (Agilent Technologies, model 5973 *inert*, Network GC system, USA) equipped with a split/splitless injector and a CTC-Multipurpose autosampler (CTC Analytics, Switzerland) with the SPME option installed. Analytes were thermally desorbed (220 °C for 2 min) from the coated fibre (polyethylene glycol fibre from Sigma–Aldrich) of the SPME in the hot injector of the GC and were separated on a Teknoram TR-150262 FFAP capillary column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m length × 250 μm internal diameter × 0.25 μm film thickness. The initial oven temperature was 35 °C held for 5 min after which the temperature was increased to 150 °C at 5 °C/min and held for 2 min. The temperature was further increased to 240 °C at 15 °C/min and held for 1 min. The split ratio was 10:1. The carrier gas was helium and the column flow rate was 1.7 mL/min.

2.6. Statistical analysis

Data was subjected to a 2-factors variance analysis (ANOVA) followed by a Bonferroni t-test to determine whether significant differences between the samples existed, using Statistica (Version 6, Statsoft (Europe) GmbH, Germany). Differences between samples with a significance level of $p \leq 0.05$ were considered as significant. Multivariate data analysis techniques were used to obtain a more comprehensible overview of the volatile aroma compounds and to investigate possible correlations amongst the analytes. Principal component analysis (PCA) was performed using *The Unscrambler* software (version 9.2.1, Camo ASA, Norway).

3. Results

3.1. Malolactic micro-vinification

Two different *O. oeni* strains were tested during MLF in two different wine matrices, a Riesling and a Chardonnay wine. The analytical parameters of the experimental wines after alcoholic fermentation are shown in Table 1.

Table 2
Duration of MLF, degree of malic acid degradation and the population of *O. oeni* R1105 and R1106.

	R1105, 98.6 g/L alcohol			R1106, 98.6 g/L alcohol			R1105, 118.3 g/L alcohol			R1106, 118.3 g/L alcohol		
	pH3.2	pH 3.6	pH 3.8	pH3.2	pH 3.6	pH 3.8	pH3.2	pH 3.6	pH 3.8	pH3.2	pH 3.6	pH 3.8
Riesling												
MLF duration (days)	30	30	23	–	30	30	sf	30	30	–	sf	61
Degree malic acid degradation (%)	97	95	100	7	99	100	24	90	97	12	36	92
Maximum population (CFU/mL)	7.17E + 06	3.59E + 06	7.87E + 06	2.74E + 06	3.60E + 06	6.97E + 06	8.20E + 05	4.57E + 06	6.47E + 06	1.13E + 05	5.60E + 06	5.10E + 06
Population last day of determination	4.72E + 06	4.07E + 05	1.11E + 05	2.22E + 04	3.00E + 06	1.47E + 06	5.37E + 03	6.73E + 05	1.18E + 06	1.37E + 02	1.00E + 04	4.50E + 04
Chardonnay												
MLF duration (days)	47	20	13	47	47	13	sf	sf	33	–	sf	33
Degree malic acid degradation (%)	93	90	99	95	92	100	52	66	100	18	65	100
Maximum population (CFU/mL)	7.94E + 06	3.00E + 06	5.68E + 06	2.32E + 06	3.28E + 06	8.90E + 06	5.73E + 06	3.88E + 06	4.40E + 06	1.68E + 06	6.96E + 06	4.40E+ + 06
Population last day of determination	3.80E + 05	3.00E + 06	2.89E + 06	1.53E + 06	6.60E + 05	2.43E + 06	9.33E + 01	1.77E + 05	9.13E + 05	2.43E + 03	6.67E + 03	9.37E + 05

–: No MLF
sf: Stuck MLF

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Duration of MLF was strain dependent and influenced by pH and ethanol content. Table 2 shows the degree of degradation of malic acid after inoculation with the bacterial strains in the Riesling and Chardonnay wines as well as the duration of MLF. Moreover, the maximum bacterial population and the population at the last day of determination are presented. Metabolism of malic acid was generally quite slow during the first days following inoculation. In the Riesling wines *O. oeni* R1105 completed MLF under almost all wine conditions, except for the wine with pH 3.2 and 118.3 g/L alcohol, within 30 days. *O. oeni* R1106 did not carry out MLF in the Riesling wines with pH 3.2. It completed MLF within 30 days in the wines with 98.6 g/L alcohol and pH 3.6 and 3.8 and in 61 days in the wine with 118.3 g/L alcohol and pH 3.8. In the wine with 118.3 g/L alcohol and pH 3.6 MLF started, but was not completed. In the Chardonnay wines *O. oeni* R1105 and R1106 completed MLF under most wine conditions, except for the wines with pH 3.2, pH 3.6 and 118.3 g/L alcohol which partially went through MLF. It took 13–47 days to complete MLF, depending on the wine condition.

O. oeni strain R1106 was more sensitive to low pH and high ethanol content. These results indicate a different behaviour in MLF performance for the two strains tested. R1105 was showing better malolactic activity with the six combinations of pH and ethanol tested. Independently from ethanol concentration (98.6/118.3 g/L), pH values 3.6 and 3.8 were found to be the best survival conditions for the strains tested. At pH 3.2, the lowest ethanol concentration was the only suitable condition for the cell survival of R1106.

3.2. Modification of free volatile aroma compounds by MLF

Various volatile components, including alcohols, esters, carbonyl compounds and acids were identified and quantified. A 2-factors variance analysis followed by a Bonferroni test was used to detect significant changes in wine composition. Tables 3 and 4 list concentrations of the volatile compounds determined in the initial wines after alcoholic fermentation and mean values of these compounds after MLF. Results show that MLF caused changes in the volatile composition of the wines.

3.2.1. Higher alcohols

Small changes were observed in the content of higher alcohols. In the Riesling wines with 98.6 g/L alcohol, the concentration of all alcohols increased when *O. oeni* strain R1105 was used. In the wines with 118.3 g/L alcohol the concentration dropped in comparison to the control (no MLF) independent of the strain used. In the Chardonnay wines on the other hand, the content of higher alcohols was higher at both alcohol concentrations compared to the control. Hexanol, associated with green and herbaceous aroma perception in wine, presented higher concentrations in the Chardonnay wines with partial and complete MLF than in the wine after alcoholic fermentation. In contrary, in the Riesling wines the content decreased after MLF especially in the wines with 118.3 g/L alcohol. One of the important wine aroma compounds 2-phenylethanol, associated with floral and rose notes presented higher concentrations in the Riesling wines with 98.6 g/L alcohol than in the ones with 118.3 g/L. The 3-methylbutanol content, on the other hand, was significantly enhanced in the Chardonnay wines with 118.3 g/L alcohol.

3.2.2. Esters

Quantitatively, levels of short-chain esters such as lactic acid ethylester in both wines and succinic acid diethylester in Riesling were the esters with the main concentration increase during MLF. Levels of these compounds were affected by the bacterial strain, wine condition and cultivar. Strain R1105 showed largest increase in lactic acid ethylester, however depending on pH and ethanol content. Wines with pH 3.2 tended to have higher concentrations than the wines with pH 3.6 and 3.8.

A significant increase of succinic acid diethylester content was observed after complete or partial MLF in the Riesling wines, especially in the treatments with pH 3.2. The smallest increase was observed in the treatments with pH 3.8.

A rise of acetic acid ethylester concentration was also detected. However, only the Chardonnay treatments showed remarkable levels at the end of MLF, as well as in the wines with stuck and partial MLF and significant differences between the bacterial strains were noted. A decrease was observed in the Riesling wines with pH 3.8.

As for other acetic acid esters, the concentration of acetic acid 3-methylbutylester, characterised by banana notes, increased following MLF in the Chardonnay wines with pH 3.6 and 3.8, while the content tended to decrease in the Riesling wines. The same tendencies were observed for acetic acid phenylethylester (floral, fruity). Acetic acid hexylester (sweet, fruity) and acetic acid 2-methyl-butylester (fruity) concentrations decreased after MLF for both strains under all tested conditions.

The production of other esters (e.g. propionic acid ethylester, i-butyric acid ethylester), increased in the wines with pH 3.2 and decreased in the wines with pH 3.6 and 3.8. Higher levels of butyric acid ethylester were detected in all Chardonnay treatments, while the levels decreased in the Riesling treatments after MLF. The tendencies were the same for both strains.

An increase in the concentration of the longer chained esters, such as hexanoic-, octanoic- and decanoic acid ethylester, was especially in the wines with 118.3 g/L alcohol observed, with final values depending on the wine parameter and cultivar.

Fig. 1 shows relative changes in various and total fruity ester concentrations.

Overall, the Chardonnay wines following MLF contained higher total fruity ester concentrations than the Riesling wines. Especially in the wines with 98.6 g/L alcohol the pH seems to influence the production of these compounds.

3.2.3. Acids

The levels of volatile fatty acids such as hexanoic, octanoic and decanoic acid, generally decreased in all the wines once MLF had finished. An increase of decanoic acid was noted in the Chardonnay wines with 98.6 g/L alcohol and pH 3.8.

3.2.4. Terpenes

The terpenes linalool (rose), cis-linalool oxide, trans-linalool oxide (flower) and α-terpineol (lilac) were only detected in the Riesling wines. A variation in the extent of the hydrolysis associated with pH was observed. In the case of cis-linalool oxide, trans-linalool oxide and α-terpineol, the glucosidase activity was lowest at pH 3.8 and highest at 3.2. Whereas with linalool, the activity was significantly lower at pH 3.2. Regarding the two bacterial strains, their contribution to terpenes was generally not significantly different.

3.2.5. Other compounds

Diacetyl was not detected or below the limit of quantification (0.5 mg/L). Enzymatic reduction might be a reason. However, acetoin was detected and results indicate that *O. oeni* R1106 produces significantly more acetoin than *O. oeni* R1105.

Moreover, decrease of acetaldehyde in all wines with successful and partial MLF was noted. All wines were also analysed for volatile sulphur compounds. No increments were detected and the amounts were below the odour detection threshold (data not shown).

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Table 3
Concentration (μg/L) of volatile aroma compounds in the Riesling wines before MLF and mean values in the wines after MLF.

	Control	R1105, 98.6 g/L alcohol			R1106, 98.6 g/L alcohol			R1105, 118.3 g/L alcohol			R1106, 118.3 g/L alcohol			Overall Trend
	pH 3.2	pH 3.2	pH 3.6	pH 3.8	pH 3.2	pH 3.6	pH 3.8	pH 3.2	pH 3.6	pH 3.8	pH 3.2	pH 3.6	pH 3.8	
Alcohols														
Hexanol	1122	1178^{a}	1117^{a}	1120^{i}	1145^{a}	1109^{a}	954^{b,c}	956^{b}	950^{b,c}	905^{c}	970^{b,c}	942^{b,c}	922^{b,c}	‡
2-Methylbutanol	32950	33587^{a}	33750^{a}	34410^{a}	32293^{a,b}	32727^{a,b}	28047^{c}	27977^{c}	28957^{b,c}	27553^{c}	26427^{c}	29283^{b,c}	29847^{b,c}	‡
i-Butanol	31460	32523^{a,b,c}	32580^{a,b}	33467^{a}	31640^{a,b,c}	31573^{a,b,c}	26327^{d}	28277^{b,c,d}	29397^{a,b,c,d}	27397^{c,d}	26803^{d}	29643^{a,b,c,d}	29997^{a,b,c,d}	‡
3-Methylbutanol	185710	190817^{a,b}	191250^{a,b}	194577^{a}	184260^{a,b,c}	184337^{a,b,c}	158920^{d}	160310^{d}	166363^{c,d}	157833^{d}	152603^{d}	168397^{c,d}	171757^{b,c,d}	‡
2-Phenylethanol	32540	34123^{a}	34777^{a}	35723^{a}	34383^{a}	36063^{a}	27777^{b}	27253^{b}	28820^{b}	28310^{b}	27410^{b}	29077^{b}	28660^{b}	‡
Esters														
Acetic acid ethylester	193000	248987^{a}	195193^{b,c,d}	176640^{c,d,e,f}	225703^{a,b}	175857^{c,d,e,f}	134397^{f,g}	157843^{b,c}	144057^{d,f,g}	111933^{g}	182527^{c,d,e}	156023^{c,d,e,f}	152003^{d,e,f,g}	‡
Acetic acid phenylethylester	315	191^{g}	323^{e,f}	355^{a,b,c}	187^{g}	332^{d,e}	363^{a,b}	179^{g,h}	350^{b,c}	368^{a}	170^{h}	308^{f}	344^{c,d}	‡
Acetic acid 3-methylbutylester	1826	828^{d}	1661^{b,c}	1972^{i}	771^{g}	1653^{b,c}	1827^{a,b}	737^{d}	1660^{b,c}	1720^{a,b,c}	628^{d}	1527^{c}	1735^{a,b,c}	↓
Acetic acid 2-methylbutylester	92	43^{b}	79^{a}	90^{g}	39^{g}	80^{a}	89^{a}	36^{b}	80^{a}	83^{a}	36^{b}	77^{a}	82^{a}	↓
Acetic acid hexylester	183	74^{c}	124^{b}	157^{a}	69^{c}	132^{a,b}	134^{a,b}	71^{c}	131^{a,b}	129^{a,b}	65^{c}	118^{b}	128^{a,b}	↓
Propionic acid ethylester	81	111^{b}	64^{c,d}	49^{e,f}	107^{b}	55^{e}	45^{e,f}	128^{a}	59^{e}	43^{f}	123^{a}	70^{e}	48^{e,f}	‡
i-Butyric acid ethylester	35	54^{b}	26^{d}	18^{g}	54^{b}	21^{g}	18^{g}	73^{a}	26^{e}	19^{g}	71^{a}	32^{e}	20^{g}	‡
Butyric acid ethylester	378	340^{a,b}	356^{a,b}	363^{a,b}	333^{b}	335^{a,b}	353^{a,b}	378^{a}	348^{a,b}	333^{b}	357^{a,b}	352^{a,b}	340^{a,b}	↓
Lactic acid ethylester	16190	44037^{a}	19650^{f}	12255^{g}	31837^{a}	17034^{c,d}	9737^{f}	10938^{e,f}	15459^{d}	9347^{f}	21830^{g}	8991^{f}	9503^{f}	↑
Succinic acid diethylester	240	1695^{b}	732^{d}	482^{f,g}	1775^{b}	624^{d,e}	393^{g}	2287^{a}	682^{d}	504^{f,g}	2388^{a}	892^{c}	554^{e,f}	↑
Hexanoic acid ethylester	1144	1007^{c,d,e}	994^{c,d,e}	968^{e}	1011^{c,d,e}	950^{e}	959^{e}	1258^{a}	1110^{c,d}	1054^{c,d,e}	1243^{a,b}	1140^{b,c}	1066^{c,d,e}	↓
Octanoic acid ethylester	1871	1805^{c}	1855^{b,c}	1751^{f}	1827^{c}	1820^{c}	1759^{e}	2192^{a}	2172^{a}	2091^{a}	2137^{a}	2044^{a,b}	2141^{a}	‡
Decanoic acid ethylester	616	538^{d}	614^{b,c,d}	578^{d}	587^{d}	614^{b,c,d}	614^{c,d}	743^{a}	781^{a}	714^{a,b}	704^{a,b,c}	702^{a,b,c}	745^{a}	‡
Acids														
Hexanoic acid	11860	11793^{a}	11837^{a}	12077^{a}	11923^{a}	12263^{a}	10170^{b}	10503^{b}	10747^{b}	10447^{b}	10470^{b}	10617^{b}	10330^{b}	↓
Octanoic acid	11000	10047^{b,c,d}	10443^{a,b,c}	10647^{a}	9953^{c,d,e}	10633^{a,b}	10327^{a,b,c}	9396^{c,e,f}	9950^{c,d,e}	9657^{d,e,f}	9243^{f}	9290^{f}	9363^{f}	↓
Decanoic acid	2620	2387^{b}	2463^{a,b}	2487^{a,b}	2540^{a,b}	2560^{a,b}	2627^{a,b}	2603^{a,b}	2727^{a}	2750^{a}	2633^{a,b}	2567^{a,b}	2390^{b}	‡
Terpenes														
trans Linalool oxide	33	54^{a}	32^{c}	27^{c,d}	52^{a}	30^{c}	22^{f,g}	47^{b}	24^{e,f}	21^{g}	49^{b}	27^{d}	22^{f,g}	‡
cis-Linalool oxide	17	32^{a}	22^{c}	19^{c,d}	32^{a}	23^{c}	18^{d,e}	28^{b}	18^{d,e}	15^{e}	31^{a,b}	19^{c,d}	15^{e}	↑
Linalool	26	15^{b}	26^{a}	26^{a}	14^{b}	25^{a}	25^{a}	14^{b}	25^{a}	25^{a}	13^{b}	25^{a}	24^{a}	↓/↔
α-Terpineol	24	37^{a}	27^{b}	24^{c,d}	36^{a}	25^{b,c}	23^{e}	36^{a}	24^{e}	21^{f}	36^{a}	25^{c,d}	21^{f}	↑/↔
Miscellaneous														
Acetoin	3753	5320^{c,d}	3310^{d}	3560^{d}	10650^{a,b}	7377^{b,c,d}	8493^{a,b,c}	3753^{d}	4167^{c,d}	4590^{c,d}	3763^{d}	4337^{c,d}	12013^{a}	↑
Acetaldehyde	31123	23282^{b,c,d}	20064^{c,d,e}	14715^{e,f,g}	27212^{a,b,c}	10938^{f,g}	6499^{g}	36505^{a,b}	15184^{d,e,f}	12092^{e,f,g}	31993^{a}	26998^{a,b,c}	10499^{f,g}	↓

Means of three replicates within rows without a common letter are significantly ($P < 0.05$) different.
The last column denotes general trend, General trend: ↑ all MLF strains increased ester concentration, ↓ ester concentration decreased, ↔/↑ ester concentration remained unchanged under some conditions but increased for others, ↔/↓ ester concentration remained unchanged under some conditions but decreased for others, ‡ strain and wine condition-specific differences

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Table 4
Concentration (μg/L) of volatile aroma compounds in the Chardonnay wines before MLF and mean values in the wines after MLF.

	Control	R1105, 98.6 g/L alcohol			R1106, 98.6 g/L alcohol			R1105, 118.3 g/L alcohol			R1106, 118.3 g/L alcohol			Overall Trend
	pH 3.2	pH 3.2	pH 3.6	pH 3.8	pH 3.2	pH 3.6	pH 3.8	pH 3.2	pH 3.6	pH 3.8	pH 3.2	pH 3.6	pH 3.8	
Alcohols														
Hexanol	811	1008^{a}	795^{a}	841^{a}	854^{a}	823^{a}	815^{a}	940^{a}	927^{a}	598^{a}	986^{a}	918^{a}	876^{a}	↑
2-Methylbutanol	21640	30067^{a}	23697^{a,b}	25220^{a,b}	22213^{b}	22450^{b}	24677^{a,b}	28850^{a,b}	30080^{a}	25843^{a,b}	29860^{a}	30110^{a}	27960^{a,b}	↑
i-Butanol	18080	26120^{a,b}	19333^{b,c}	22167^{a,b,c}	19317^{b,c}	18343^{c}	21720^{a,b,c}	26030^{a,b}	27170^{a}	24197^{a,b,c}	26680^{a}	27600^{a}	25653^{a,b}	↑
3-Methylbutanol	154420	220063^{a}	180143^{a,b}	184843^{a,b}	164010^{b}	168113^{a,b}	180957^{a,b}	211230^{a,b}	219950^{a}	197889^{a,b}	219530^{a}	228280^{a}	205263^{a,b}	↑
2-Phenylethanol	20620	19557^{a}	18223^{a,b}	19340^{a,b}	15980^{b}	18640^{a,b}	19340^{a,b}	18890^{a,b}	20020^{a}	19617^{a}	20060^{a}	19680^{a}	19607^{a}	↓
Esters														
Acetic acid ethylester	82390	187233^{a}	183337^{a}	162527^{a}	100977^{a}	132987^{a}	157637^{a}	156560^{a}	187850^{a}	164900^{a}	150570^{a}	186280^{a}	182563^{a}	↑
Acetic acid phenylethylester	344	249^{d}	340^{a,b}	335^{a}	269^{c,d}	302^{b,c}	360^{a}	261^{c,d}	297^{b,c}	290^{c,d}	264^{c,d}	251^{c,d}	279^{c,d}	↓
Acetic acid 3-methylbutylester	3815	3528^{d,e}	4975^{a}	4660^{a,b}	3300^{e}	4128^{b,c,d}	4641^{a,b}	3851^{c,d,e}	4255^{a,b,c,d}	4325^{a,b,c}	3596^{c,d,e}	4078^{b,c,d}	4259^{a,b,c,d}	↑
Acetic acid 2-methylbutylester	143	121^{d}	173^{a}	163^{a,b}	117^{d}	137^{b,c,d}	169^{a}	128^{c,d}	149^{a,b,c,d}	154^{a,b,c}	128^{c,d}	134^{a,b,c,d}	144^{a,b,c,d}	↕
Acetic acid hexylester	292	166^{d}	266^{a,b,c}	285^{a,b}	190^{d}	220^{b,c,d}	300^{a}	202^{c,d}	227^{b,c,d}	233^{a,b,c,d}	199^{c,d}	217^{b,c,d}	206^{c,d}	↓
Propionic acid ethylester	47	124^{a}	56^{e}	49^{e}	102^{b}	57^{e}	46^{e}	126^{a}	83^{b,c}	62^{e}	132^{a}	82^{c,d}	63^{c,e}	↑
i-Butyric acid ethylester	5	20^{b,c}	5^{fg}	3^{g,h}	18^{c}	7^{ef}	2^{h}	22^{a,b}	9^{d,e}	5^{fg}	23^{a}	10^{d}	5^{fg}	↑,↕
Butyric acid ethylester	376	455^{a}	443^{a,b}	440^{a,b}	438^{a,b}	405^{b}	435^{a,b}	450^{a,b}	449^{a,b}	433^{a,b}	453^{a,b}	428^{a,b}	431^{a,b}	↑
Lactic acid ethylester	10850	197533^{a}	67803^{c,d,e}	47332^{c,d,e}	124433^{b}	83200^{b,c,d}	44320^{d,e}	99100^{b,c}	65280^{c,d,e}	55107^{c,d,e}	24940^{e}	67530^{c,d,e}	64150^{c,d,e}	↑
Hexanoic acid ethylester	1050	892^{b}	1050^{a,b}	1059^{a,b}	948^{a,b}	947^{a,b}	1060^{a,b}	1137^{a}	1104^{a,b}	1072^{a,b}	1161^{a}	1095^{a,b}	984^{a,b}	↕
Octanoic acid ethylester	1530	1088^{b}	1528^{a,b}	1711^{a}	1301^{a,b}	1464^{a,b}	1660^{a}	1529^{a,b}	1644^{a}	1561^{a,b}	1582^{a,b}	1633^{a,b}	1488^{a,b}	↕
Decanoic acid ethylester	372	349^{a}	470^{a}	499^{a}	409^{a}	431^{a}	486^{a}	449^{a}	478^{a}	471^{a}	444^{a}	468^{a}	450^{a}	↑
Acids														
Hexanoic acid	13380	12390^{a,b,c}	11730^{b,c}	12789^{a,b,c}	11655^{c}	12269^{a,b,c}	12803^{a,b,c}	12780^{a,b,c}	13120^{a,b}	13147^{a}	13230^{a}	12900^{a,b,c}	12857^{a}	↓
Octanoic acid	11650	10317^{b,c,d}	10983^{a,b,c}	11860^{a}	11195^{c,d,e}	10780^{a,b}	11827^{a,b,c}	10750^{c,f}	10390^{c,d,e}	10840^{d,e,f}	10570^{f}	10580^{f}	10187^{f}	↓
Decanoic acid	1770	1613^{b}	2040^{a,b}	2323^{a,b}	2230^{a,b}	1953^{a,b}	2290^{a,b}	1780^{b}	1610^{b}	1913^{a,b}	1680^{b}	1700^{a}	1733^{b}	↕
Miscellaneous														
Acetoin	2887	3160^{b}	n.q.	n.q.	12297^{a}	10107^{a}	4717^{b}	n.q.	2697^{b}	n.q.	n.q.	4767^{b}	n.q	↑
Acetaldehyde	32275	16110^{a,b}	14436^{b}	22001^{a,b}	12864^{b}	11507^{b}	16195^{a,b}	25223^{a,b}	17345^{a,b}	35076^{a}	34480^{a}	12106^{a}	24160^{a,b}	↓

Means of three replicates within rows without a common letter are significantly ($P < 0.05$) different.
The last column denotes general trend. General trend: ↑ all MLF strains increased ester concentration, ↔/↑ ester concentration remained unchanged under some conditions but increased for others, ↓ ester concentration decreased, ↕ strain- and wine condition-specific differences.
n.q. - not quantifiable (traces).

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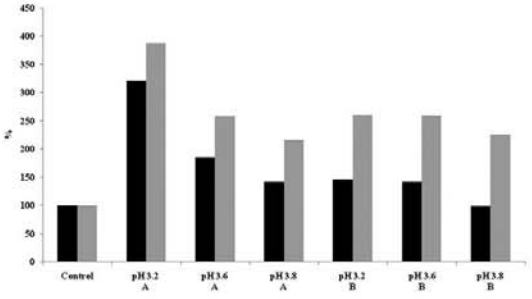

Fig. 1. Relative changes in total ester concentration in Riesling- (■) and Chardonnay- (■) wines fermented with *O. oeni* R1105 at 98.6 g/L alcohol (A) and 118.3 g/L alcohol (B).

As an overview of the results, a principal component analysis (PCA) of the volatile aroma compounds of the Riesling wines was performed. 99% of the variance was explained by the first two principal components. As shown in Fig. 2, these PCA's separated the samples according to pH and ethanol concentration. On the score plot separation along PC1 was associated with discrimination of treatments with pH 3.2 from treatments with pH 3.6 and 3.8. Loadings for succinic acid diethylester were correlated with treatments pH 3.2, while loadings for acetic acid 3-methylbutylester were correlated with treatments pH 3.6 and 3.8. Higher loadings for longer chained esters were correlated with wines with 118.3 g/L alcohol along PC2, while loadings for hexanol were correlated with wines with 98.6 g/L alcohol. Similar results were obtained for the Chardonnay wines (Fig. 3).

Discussion

The selection of bacterial strains for the vinification process is principally based on the culture's compatibility with the wine environment and the consumption of malic acid. Various studies have investigated the evolution of MLF in wine, with respect to high ethanol concentration, low pH or SO_2 (Alegria, Lopez, Ruiz, Saenz, Fernandez, Zarazaga et al., 2004; Carrete, Reguant, Rozes, Constanti, & Bordons, 2006; Carrete, Vidal, Bordons, & Constanti, 2002; Gockowiak & Henschke, 2003; Guzzon et al., 2009; Rosi, Fia, & Canuti, 2003; Solieri, Genova, Paola, & Giudici, 2010)Currently, climate change and vinification practises frequently result in wines with higher ethanol concentrations (>102.57 g/L) (Mira de Orduña, 2010). The ethanol content and pH are crucial factors limiting bacterial growth and activity. Solieri et al. (2010) showed that low pH values are the most negative attribute influencing the malolactic activity. Gockowiak and Henschke (2003) observed that pH in the range 2.9–3.5 had a generally large influence on bacterial viability and MLF, whereas alcohol concentration in the range of 98.6–114.4 g/L had a lesser impact. Moreover, the wine matrix was proven to be an important oenological factor that can affect bacterial growth and MLF. While a pH of 3.5 allowed MLF accomplishment by three out of four strains independently from ethanol content, pH values of 3.0 and 3.2, combined with 78.9 and 102.6 g/L ethanol respectively, were inhibitory for all tested strains (Solieri et al., 2010). This correlates with results from the present study where MLF was as well faster at lower selective pressure. Moreover, *O. oeni* strain R1105 showed greater resistance to high ethanol content and low pH values.

Previous studies have demonstrated the ability of *O. oeni* to modify various fermentation-derived volatile compounds, organic acids and amino acids. However, it was shown that changes in the aroma profile of wine following MLF are not only affected by LAB strain, but also by wine composition, cultivar, vintage and wine-growing region. Moreover, not only biochemical, but also chemical reactions take place during that time. There is a number of reports showing changes in the volatile aroma profile of wines after MLF, summarised by Sumby, Grbin, and Jiranek (2010). The present study in Riesling and Chardonnay wines has demonstrated that bacterial modifications of ester concentrations are affected by various factors such as wine composition, bacterial strain and cultivar. Results point out that enzymatic actions are present even if the MLF is not conducted successfully, which suggests that partial MLF, can already contribute to the wine aroma. Thus it was observed, that for example Chardonnay and Riesling treatments, which only have undergone partial MLF, showed never the less increases in fruity esters such as acetic acid ethylester, propionic acid ethylester and butyric acid ethylester.

In the present work the concentration of some of the volatile aroma compounds (e.g. acetic acid ethylester, acetic acid 3-methylbutylester, succinic acid diethylester or lactic acid ethylester) appeared to be influenced especially by wine pH and ethanol

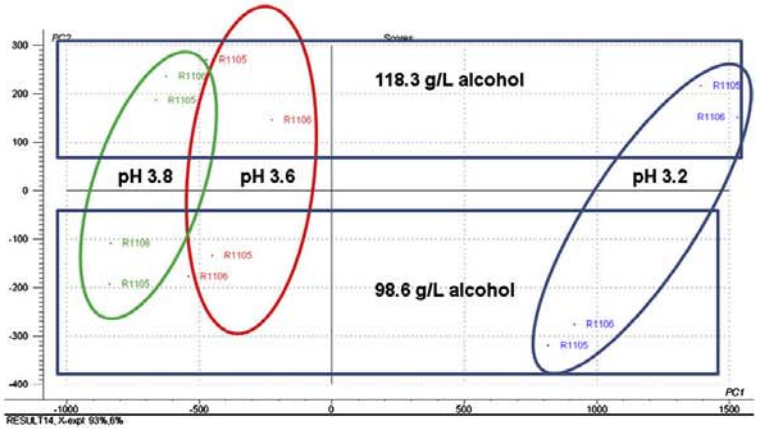

Fig. 2. PCA score plot derived from volatile compounds of all Riesling wines following MLF.

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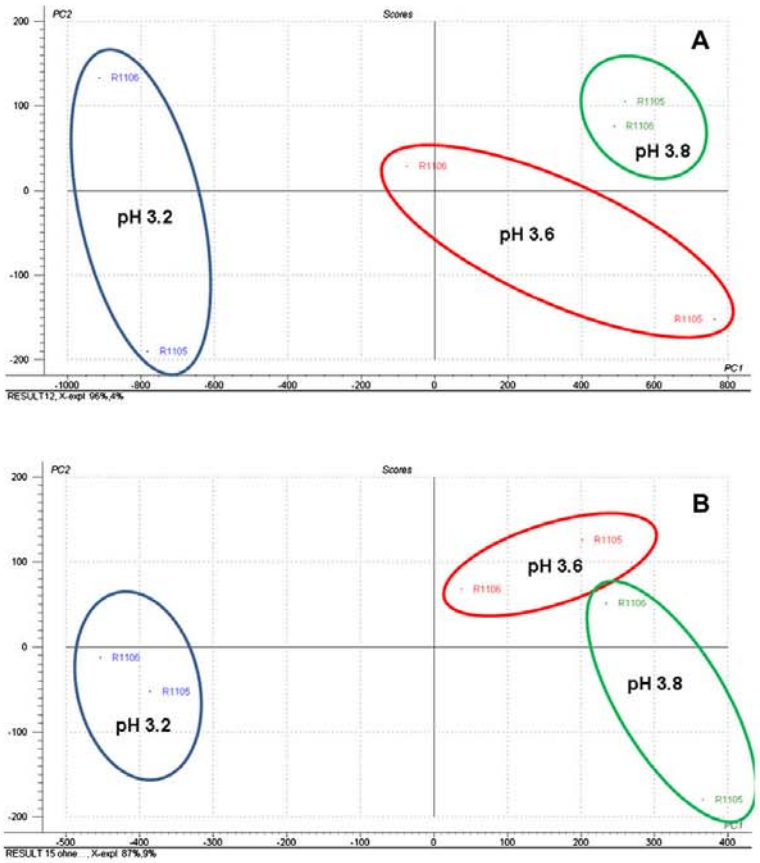

Fig. 3. PCA score plot derived from volatile compounds of all Chardonnay wines following MLF with 98.6 g/L alcohol (A) and 118.3 g/L alcohol (B).

content. In general, MLF treatments in Chardonnay produced higher concentrations in total esters and total alcohols. Bartowsky et al. (2010) observed changes in the volatile fermentation-derived compounds at different wine pH (3.3 and 3.7) in red wines. A lower pH resulted in greater increases in total fruity esters which were reflected in sensorial analysis. In the present work, these observations were confirmed for the white wines Riesling and Chardonnay.

Levels of short-chain esters, such as lactic acid ethylester, acetic acid ethylester and succinic acid diethylester, presented higher concentrations after complete MLF but also in the wines with partial or stuck MLF. Lactic acid ethylester and acetic acid ethylester were quantitatively the predominant esters in all treatments. Lactic acid ethylester, associated with fruitiness, milky notes and an increased mouthfeel, is an important aroma compound produced during MLF. *O. oeni* R1105 tended to produce more lactic acid ethylester than *O. oeni* R1106. Increased concentration of this ester and strain dependency was also observed by others (Boido et al., 2009; Maicas et al., 1999; Pozo-Bayón, G-Alegria, Polo, Tenorio, Martin-Alvarez, CalvodelaBanda et al., 2005; Ugliano & Moio, 2005).

The concentrations of acetic acid ethylester were higher in the Riesling than in the Chardonnay wines. However, only the Riesling wines at pH 3.2 and 98.6 g/L ethanol exceeded 200 mg/L which can affect the wine aroma with undesirable 'solvent' characters, when levels exceed 200 mg/L (Dittrich, 1983). At lower concentrations it adds to wine complexity and contributes fruity aromas.

Succinic acid diethylester, associated with fruity and floral notes, contributes significantly to the wine aroma (Clarke & Bakker, 2004) and previous studies reported an increase during ageing (Jackson, 2000). However, other authors observed increased concentrations after MLF at wine pH 3.2 to 3.7, depending on bacterial strain used (Boido et al., 2009; Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985; Herjavec, Tupajić, & Majdak, 2001; Lee, Hwang, Lee, & Hong, 2009; du Plessis, Steger, du Toit, & Lambrechts, 2002; Ugliano & Moio, 2005). In our study, the highest content of succinic acid diethylester was found in the wines with pH 3.2, also in the wines with partial MLF where the bacterial population was still present at cfu/mL of 10^2 to 10^4.

The production of other esters, which contribute to pleasant fruity notes, showed significant differences after MLF depending on wine condition and strain. The total amounts of esters found after MLF in the Riesling and Chardonnay wines suggest their beneficial contribution to the wine's final aroma. Bartowsky, Costello, and McCarthy (2008) observed that ethylesters tend to increase and acetic acid esters decrease following MLF in red wines. These findings were in agreement with Ugliano and Moio (2005). The

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outcome of this study indicates that the rise or decline of these esters is wine pH dependent. At pH 3.2 an increase of ethyl-and acetic acid esters was observed, whereas with rising pH a decrease was noted. The Chardonnay wines showed an increase in ethyl and acetic acid esters after bacterial inoculation. The Riesling wines, however, had an overall trend of decreasing acetate and ethylester concentrations, especially at 118.3 g/L alcohol.

In this study, the content of the terpenes trans- and cis-linalool oxide and α-terpineol increased with lower pH values and the linalool content increased with higher pH. The decrease of linalool at low pH may be due to rearrangements of linalool into α-terpineol under the pH condition of wine (Di Stefano, 1989; Gunata, Bayonove, Baumes, & Cordonnier, 1986). Ugliano, Genovese, and Moio (2003) also observed a significant increase in the concentration of total free terpenols at low pH. Latter authors noted that the hydrolysis of aroma precursors appeared to be strongly enhanced by the occurrence of MLF by comparing the fermented with the unfermented samples which contained low concentrations of terpenols, probably as a result of slow acid catalysed hydrolysis. In the Riesling treatments with stuck or no MLF, the terpenes trans-linalool oxide, cis-linalool oxide and α-terpineol were also present in higher concentrations. Similar observations were made by Hernandez-Orte et al. (2009) in a model wine system enriched in glycosidic precursors. Their results indicated that *O. oeni* and *Lactobacillus* strains were able to induce clear changes on the volatile profile derived from grape flavour precursors, although only the *O. oeni* strains conducted MLF. They suggested that MLF metabolism may not be linked to the abilities of LAB to hydrolyse and release glycosidically bound aroma compounds.

The acetaldehyde content decreased in the wines inoculated with bacteria. These findings were also observed by others (Mayer, Pause, & Vetsch, 1976; Nielsen & Richelieu, 1999; Osborne, Dube Morneau, & Mira de Orduna, 2006; Pozo-Bayón et al., 2005) and it was proposed that the degradation of this compound and other aldehydes during MLF may cause a reduction in herbaceous and green aroma (Osborne, Mira de Orduña, Pilone, & Liu, 2000).

This work confirms that *O. oeni* can contribute significantly to the formation of volatile aroma compounds in white wines and that partial MLF already has distinct influences on the aroma profiles. However, it appears that pH and ethanol content are also important factors influencing the volatile aroma composition. Wine composition and desired outcome of the wine are important criteria for the correct choice of *O. oeni* strain for MLF, as it influences the final chemical composition and sensory profile of wine. Research has shown that LAB possess a broad range of ester synthesising and hydrolysing activities, many of which may affect wine composition and organoleptic properties. A better understanding of these bacterial activities is of great interest to the wine industry and new techniques for altering wine aroma could be developed.

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2.2 Cool climate Riesling wines: Impact of different malolactic fermentation inoculation scenarios on wine aroma

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Cool climate Riesling wines: Impact of different malolactic fermentation inoculation scenarios on wine aroma

Running title: Low pH Riesling wines, MLF and the aroma profile

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Abstract

BACKGROUND: During malolactic fermentation (MLF), lactic acid bacteria influence wine aroma and flavour by the production of volatile metabolites and the modification of aroma compounds derived from grapes and yeasts. The present study investigated the impact of different MLF inoculation strategies with two different *Oenococcus oeni* strains on cool climate Riesling wines and the volatile wine aroma profile. Four different timings were chosen for inoculation with bacteria to conduct MLF in a Riesling must / wine with high acidity (pH 2.9 – 3.1).

RESULTS: Treatments with simultaneous inoculation showed a reduced total fermentation time (alcoholic and malolactic) compared to the sequential inoculations. No negative impact of simultaneous alcoholic and malolactic fermentation on fermentation success and on the final wine volatile aroma composition was observed. Compared to sequential inoculation, wines with co-inoculation tended to have higher concentrations of ethyl and acetate esters, including acetic acid phenylethylester, acetic acid 3-methylbutylester, butyric acid ethylester, lactic acid ethylester and succinic acid diethylester.

CONCLUSION: Applying a co-inoculation protocol may offer microbiological, technological and sensorial advantages, especially in low-pH, cool-climate white musts with potential high alcohol content.

Keywords: Malolactic fermentation; co-inoculation; low pH wine; *Oenococcus oeni*; volatile aroma

INTRODUCTION

Malolactic fermentation (MLF), the enzymatic decarboxylation of L-malic acid to L-lactic acid and carbon dioxide, is the important secondary fermentation conducted by lactic acid bacteria (LAB), with *Oenococcus oeni* being the most tolerant bacterial species to the harsh environmental wine conditions and the most desired bacterium to carry out this fermentation (Mills et al. 2005; Fugelsang and Edwards 2007). MLF is known to improve the wine quality through deacidification, enhancement of microbial stability and production of volatile aroma compounds (Davis et al. 1985; Lonvaud-Funel 1999). The success is influenced by several oenological parameters, such as pH, temperature, alcohol content and sulphur dioxide (SO_2) concentration (Lerm et al. 2010). In addition to these parameters, the presence of some yeast inhibitory metabolites such as medium chain fatty acids (Alexandre et al. 2004) or peptic fractions (Nehme et al. 2010) can affect bacterial viability and MLF. Due to these possible antagonistic or undesirable interactions between yeast and bacteria, the correct choice and combination of yeast and bacterial strains is important for the success of MLF.

There are different MLF inoculation possibilities, such as simultaneous inoculation for alcoholic and malolactic fermentation (co-inoculation) of yeasts and LAB, inoculation during alcoholic fermentation (AF) and inoculation after the completion of AF (sequential inoculation) (Henick-Kling 1993; Fugelsang and Edwards 2007). Simultaneous inoculation can be an efficient alternative to overcome potential inhibition of LAB, due to high ethanol concentrations and reduced nutrient content (e.g. essential amino acids, vitamins or minerals) (Jussier et al. 2006; Zapparoli et al. 2009). Hence, a more successful induction of MLF due to a gradual adaption of bacteria to increasing alcohol concentrations and due to the benefit from higher nutrient availability present in the must, compared to the condition at the end of AF (Rosi et al. 2003). Likewise, simultaneous inoculation of musts or wines with high acidity but still low levels of ethanol and higher nutrient concentration may help to avoid potential MLF problems. It also would be beneficial regarding technical aspects: wines after successful co-inoculation would be immediately ready for downstream treatments, such as racking, fining, and SO_2 addition, thus increasing microbiological stability and processing efficiency (Jussier et al. 2006). Various studies have been conducted to determine the best moment and condition for bacterial inoculation (Henick-Kling and Park 1994; Semon et al. 2001; Rosi et

al. 2003; Jussier et al. 2006; Massera et al. 2009). Jussier et al. (2006) and Massera et al. (2009) observed no negative effects on fermentation success and kinetics, linked with simultaneous inoculation, compared to sequential inoculation and no difference in the final quality of Chardonnay and Malbec wines. The results pointed out the reduction of total fermentation time and a better control of MLF. Rosi et al. (2003) observed that pH and timing of bacterial inoculation were critical to how fast MLF starts. Low pH in a commercial white grape juice had a negative effect on bacterial viability; additionally inoculation halfway through AF caused a bacterial reduction (Rosi et al. 2003).

Most of these studies have concentrated on the microbial interactions, bacterial viability, and only a few wine parameters, such as sugar, malic, citric or acetic acid levels. During co-inoculation, the simultaneous metabolism of glucose and citric acid by *O. oeni* could result in higher acetic acid concentrations (Davis et al. 1985; Liu 2002). It has also been observed, that wines that have undergone simultaneous AF/MLF tend to be less buttery and are fruitier with slightly higher but sensorial insignificant levels of acetic acid (Henick-Kling 1993; Bartowsky et al. 2002; Jussier et al. 2006; Krieger 2006; Massera et al. 2009). However, little is known about the influence of the LAB inoculation timing on the volatile aroma composition of the wine, especially in cool climate Riesling wines.

The bacterial strain, metabolic activity, as well as the kinetics of MLF, will influence the sensory profile of the wine linked to the vinification techniques, the physical and chemical composition of the wine. Research, mostly carried out in synthetic wine model solutions, Chardonnay or red wines (e.g. Merlot, Cabernet Sauvignon, Tannat), has shown that LAB have the potential to alter the aroma profile of wine by the production of volatile secondary metabolites or the modification of grape and yeast derived metabolites including ethyl esters, acetate esters, acids and alcohols (de Revel et al. 1999; Lonvaud-Funel 1999; Maicas et al. 1999; Hernandez-Orte et al. 2009; Bartowsky et al. 2010). Many of these alterations are strain dependent, however the vinification technique can also affect the final wine aroma profile and these flavour impacts of individual bacterial strains are also of great interest for winemakers.

Due to the fact that induced MLF is often difficult to achieve in wines with high acidity, the development of strategies to favour a biological deacidification of low pH wines is important to prevent sluggish or stuck fermentation. The present study investigated the

impact of different inoculation scenarios with two different *O. oeni* strains on the kinetics of MLF in low pH Riesling must and wine and its effect on the volatile wine aroma profile. Four different timings were chosen for inoculation with bacteria to conduct MLF. A cool-climate Riesling was chosen as a typical example of a white wine with high acidity.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

The *O. oeni* strains R1105 and R1124 (Lallemand, Toulouse, France) were obtained as freeze dried cultures. MRS-T agar (MRS agar containing 10 % tomato juice, pH 5) was used for bacterial growth determination. The medium contained 50 mg L^{-1} Actistab (DSM Food Specalities Dairy Ingredients, Delft, The Netherlands) dissolved in ethanol which inhibited moulds and yeasts. The bacterial cell numbers were determined weekly during MLF. Plates were incubated anaerobically at 30°C for 7 days. Colony counts were carried out and reported as colony-forming units per mL (CFU mL^{-1}).

Micro-vinification

Riesling grapes from the Rheingau wine region (Germany) were harvested during the 2010 season. The grapes were destemmed and crushed and a standard addition of 30 mg L^{-1} sulphite (in form of potassium bisulphite) was added. The must was then settled over night and pasteurised at 82°C for 20 s. The chemical composition of the must is shown in Table 1.

Table 1. Analytical parameters of the initial must and the wines at 40%, 60% of AF and after completion of AF (EndAF)

Wine	pH	Acetic acid (g L^{-1})	Total acidity (g L^{-1})	Malic acid (g L^{-1})	Lactic acid (g L^{-1})	Total sugar (g L^{-1})	Ethanol (% v/v)
Must	3.1	n.q.[a]	15	6.5	n.q.	218.1	n.d.[b]
40% of AF	2.9	0.5	14.1	6	n.q.	124.9	5.4
60% of AF	3	0.6	13.9	5.9	n.q.	108.4	6.4
End AF	3.1	0.8	11.8	5.3	n.q.	1.5	13.1

[a] n.q., not quantifiable (limit of quantification 0.1 g L^{-1})

[b] n.d., not detected.

After the pH had been adjusted to 3.1 with 5 M NaOH, the must was inoculated with the yeast strain Uvaferm GHM® (Lallemand, Germany) in combination with two different LAB starter cultures and four different inoculation strategies, namely (A) inoculation of LAB starter cultures 24 h after yeast addition; (B) inoculation at 40 % of AF; (C) inoculation at 60 % of AF and (D) inoculation after the completion of AF (residual sugar < 5 g L^{-1}). The yeast was rehydrated beforehand using Go-Ferm® (Lallemand, Germany) according to the manufactures recommendations. Both *O. oeni* strains (R1105, R1124) were rehydrated and pre-acclimatised using the 1-Step® protocol (Lallemand) following the manufactures instructions. Both, yeast and bacterial strains were inoculated with approximately 10^6 CFU mL^{-1}. All fermentations were carried out in green 2 L bottles in triplicate at 20°C. The wine with sequential MLF was racked at the end of AF, divided into the 2 L bottles and then inoculated with the bacterial strains.

Alcoholic fermentation was monitored by sugar depletion. The wines were considered to be dry and AF concluded when the reducing sugar level was below 5 g L^{-1}. MLF was monitored by malic acid degradation and lactic acid production. MLF was considered complete when malic acid concentration was less than 0.2 g L^{-1}. To each wine sample, 80 mg L^{-1} of sulphite were added immediately upon completion of MLF. All samples were cold stabilised at 4°C and bottled without filtration and without prior addition of further fining agents.

Analysis of must and wine for organic acids

Must was analysed before inoculation and samples were collected during and after AF and MLF for organic acids (tartaric acid, malic acid, lactic acid, acetic acid and citric acid) using high performance liquid chromatography (HPLC). HPLC analysis was performed according to Schneider et al. (1987) with following modifications: 5 µL of sample were injected into the Agilent Technologies 1100 series liquid chromatograph equipped with a multiwavelength detector (MWD) and analysed using an Allure® Organic Acid column (250mm x 4.6 mm inside diameter) (Restek GmbH, Bad Homburg, Germany) with a Security Guard™ Cartridge C18 4 x 3 mm (Phenomenex, Aschaffenburg, Germany). The eluent was distilled water with 0.0139 % sulphuric acid and 0.5 % ethanol. The column was operated at 46 °C with an eluent flow rate at 0.6 mL/min.

Eluting compounds were detected by UV absorbance at 210 nm. Citric acid was also measured enzymatically (Boehringer, Mannheim, Germany). Must components are reported as a single value without standard deviation. All other analyses are reported as the means of three determinations (one for each trial carried out in triplicate).

Gas chromatography - mass spectrometry (GC-MS) Analysis for volatile aroma compounds

Higher alcohols, esters, volatile fatty acids and terpenes were analysed using GC-MS. The analysis was performed using a GC Hewlett Packard (HP) 5890 Series II (Agilent, Santa Clara, USA), coupled to a 5972 HP Mass Selective Detector (Agilent). The GC was fitted with a cooled injection system (CIS 3) (Gerstel GmbH, Mülheim, Germany). Compounds were separated on a Varian VF-5MS column (Palo Alto, USA) with dimensions of 60 m x 0.32 mm x 1 µm. Analysis was done according to Rapp et al. (1994), modified by Fischer and Rauhut (2005, unpublished) with the following conditions: injection was splitless (1 min) with the injector start temperature of 30°C and then increased to 230°C at 12°C/min, and held for 4 min. The initial oven temperature was 40 °C, held for 5 min, then increased to 125 °C at 3 °C/min, further increased to 200 °C at 6 °C/min and held for 14.2 min. Helium was used as carrier gas at a constant flow rate (1 mL/min). The mass spectrometer was set to scan mode, covering a mass-to-charge ratio range (m/z) from 35 to 250 atomic mass units (amu). The temperature of the MS was set to 180°C, respectively.

Data analysis

Data was subjected to one-way analysis of variance (ANOVA) followed by Tukey's studentised range (HSD) test to determine whether significant differences between the samples existed, using the SAS Enterprise Guide 4.1 (version 9.1.3, Procedure PROC GLM, SAS Institute, Germany). Differences between samples with a significance level of 5% ($p \leq 0.05$) were considered as significant. Multivariate data analysis techniques were used to obtain a more comprehensible overview of the volatile aroma compounds and to investigate possible correlations amongst the analytes (Naes et al. 2002). Princi-

pal component analysis (PCA) was performed using *The Unscrambler* software (version 9.2.1, Camo ASA, Norway).

RESULTS

Two *O. oeni* strains and four different MLF inoculation scenarios were evaluated in a cool climate Riesling must fermented with one yeast strain.

Impact on alcoholic fermentation

Alcoholic fermentation was completed in 13 or 14 days in all experiments independently of the timing of bacterial inoculation at 24 h, 40% (day 2) and 60% (day 3) of the AF (Figure 1). The wine inoculated with LAB after completion of AF (EndAF), took as long for the AF as the other treatments inoculated simultaneously. The analytical parameters of the experimental wines at 40%, 60% of AF and after completion of AF are shown in Table 1.

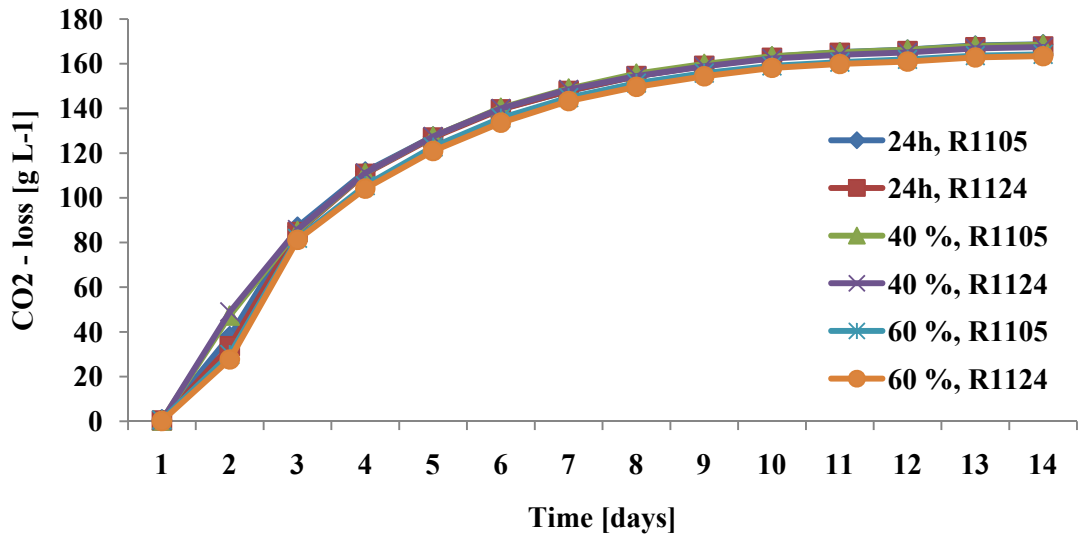

Figure 1. Average alcoholic fermentation process of the treatments with bacterial inoculation at 24h, 40% (day 2) and 60% (day 3) of AF.

Effect of bacterial inoculation timing on MLF

Treatments with simultaneous inoculation showed a reduced total fermentation time (AF+MLF) compared to the sequential inoculations. Only, in the 24h treatment inoculated with R1124 the length of MLF itself in was longer than its respective sequential treatment.

The 24 h treatment was inoculated for MLF at day one of AF, the 40% treatment at day two and the 60% treatment at day three of AF. The 24 h treatment inoculated with *O. oeni* R1105 took 49 days to complete MLF. *O. oeni* R1124 took 77 days. The 40 % treatment inoculated with *O. oeni* R1105 took 62 days to conduct MLF and *O. oeni* R1124 68 days. In the 60 % treatment MLF was completed in 49 days (Figure 2 and 3). The sequential inoculations concluded MLF in 70 to 84 days (Figure 4).

When bacteria were inoculated after 24h and at 60% of AF, the malic acid decrease began between day 8 and 15 of AF. The population of *O. oeni* R1105 dropped to 10^5 CFU mL^{-1} at both inoculation times. The population of R1124 decreased as low as 10^3 CFU mL^{-1} in the 24 h treatment and to 10^4 CFU mL^{-1} in the 60 % treatment. When bacteria were inoculated at 40% of AF, the population of both strains dropped to 10^4 CFU mL^{-1} and it took 14 to 18 days till malic acid decrease started. From the analytical data recorded in Table 1 it is evident that at 40% of AF the pH was lower compared to the initial value and at 60% of AF. In the wines with sequential inoculation the bacterial population dropped to 10^3 CFU mL^{-1} and malic acid degradation commenced after approximately 18 days.

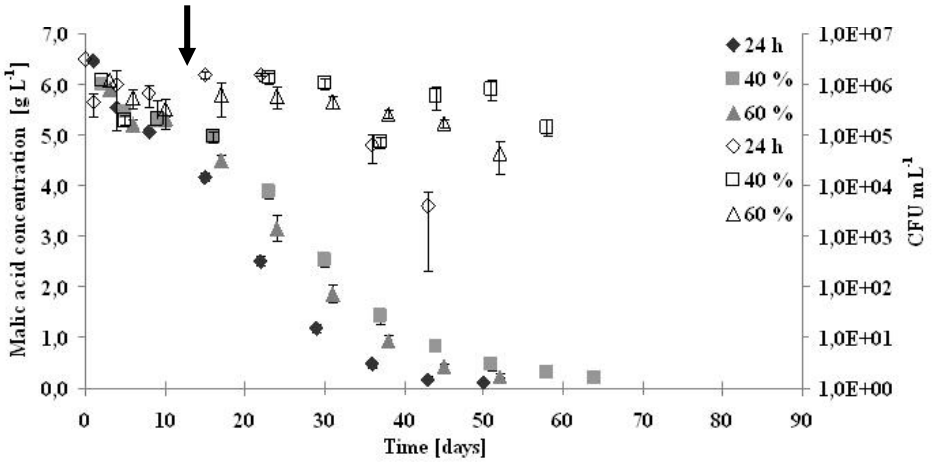

Figure 2. Cell numbers (CFU mL^{-1}) of *O. oeni* R1105 (open symbols) and mean values and standard deviation of L-malic acid concentration (filled symbols). Arrow indicates end of AF.

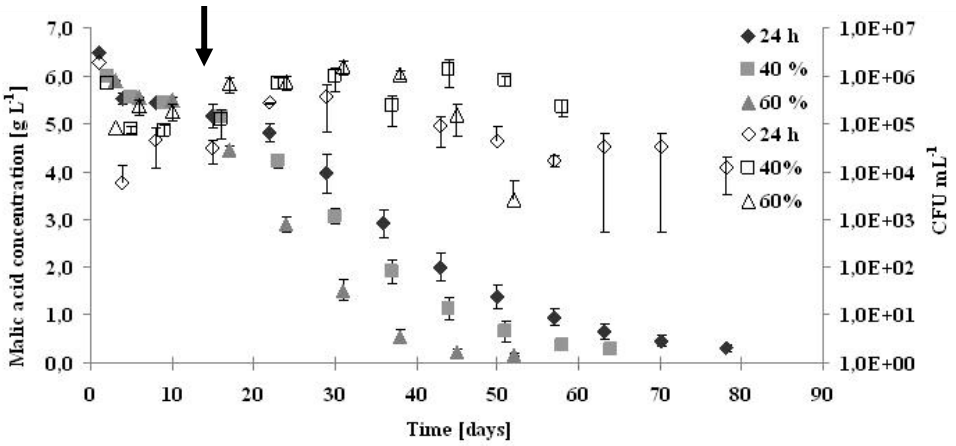

Figure 3. Cell numbers (CFU) of *O. oeni* R1124 (open symbols) and mean values and standard deviation of L-malic acid concentration (filled symbols). Arrow indicates end of AF.

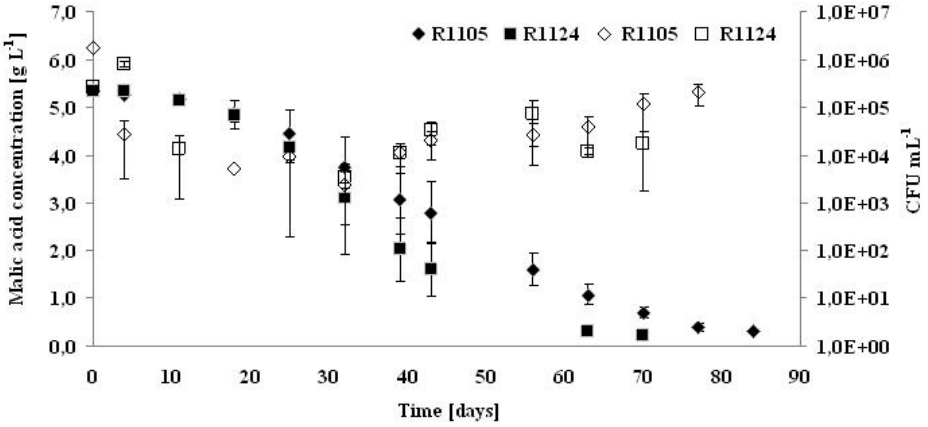

Figure 4. Cell numbers (CFU) of *O. oeni* R1105 and R1124 (open symbols) and mean values and standard deviation of L-malic acid concentration (filled symbols) during malolactic fermenation in the treatment with sequential MLF inoculation.

The volatile acidity increased to 0.6 – 0.7 g L^{-1} after MLF. However, the concentration in the fermenting musts before inoculation with *O. oeni* was already 0.5 - 0.6 g L^{-1} and in the wine at the end of AF 0.8 g L^{-1} (Table 1, 2).

The citric acid, initially present in the grape juice at concentration of 0.25 g L^{-1}, was completely utilized by *O. oeni* R1124 in all treatments. *O. oeni* R1105 only partially degraded citric acid in all treatments (Table 2).

Table 2. Concentration (g L^{-1}) of acetic acid and citric acid in the wines after MLF

	R1105				R1124			
	24h	**40%**	**60%**	**AFEnd**	**24h**	**40%**	**60%**	**AFEnd**
Acetic acid	0.61 ± 0.02	0.61 ± 0.01	0.66 ± 0.04	0.73 ± 0.02	0.66 ± 0.01	0.66 ± 0.03	0.68 ± 0.04	0.77 ± 0.01
Citric acid	0.14 ± 0.02	0.15 ± 0.03	0.17 ± 0.01	0.11 ± 0.01	n.q.[a]	n.q.	n.q.	n.q.

[a]n.q., not quantifiable (limit of quantification 0.1 g L^{-1})

Modification of free volatile aroma compounds by MLF

Various volatile components, including alcohols, esters and acids were identified and quantified. A one way analysis of variance as well as PCA were performed in order to observe underlying trends in the data and to obtain more information about variations in wine composition as a result of different bacterial strains and to compare the influence of the different inoculation timings on the volatile aroma composition. Table 3 lists concentrations of the volatile compounds determined in the wines after alcoholic fermentation and mean values of these compounds after MLF. Alphabetical letters indicate significant differences among the mean values obtained for each strain that performed MLF. Results show that MLF and inoculation timing as well as bacterial strains caused different and significant changes in the volatile aroma composition of the wines.

Higher alcohols

The concentration of total higher alcohols increased in most treatments after MLF except in the treatment inoculated after 24 h with R1105 and the treatment inoculated at 60 % of AF with R1124. The content of hexanol, 3-methylbutanol and 2-phenylethanol increased while the concentration of 2-methylbutanol decreased after MLF.

Esters

The content of all acetate esters, except for acetic acid ethylester, decreased after MLF in all treatments. Compared to the co-inoculation, the treatments with sequential inoculation had the lowest concentration of acetic acid phenylethylester (floral, fruity aroma) and acetic acid 3-methylbutylester (banana odour). Moreover, in the wines fermented with *O. oeni* R1105, higher concentrations of acetic acid 2-methylbutylester and acetic acid 3-methylbutylester were detected.

Table 3. Concentration (µg L^{-1}) of volatile aroma compounds in the Riesling wines before and after MLF

	Wine without MLF	After MLF																Overall Trend[a]
		R1105								R1124								
		24h		40%		60%		AFEnd		24h		40%		60%		AFEnd		
MLF duration in days	-	49		62		49		77		77		68		60		70		
Alcohols																		
Hexanol	540	681 ± 86	A	608 ± 36	AB	629 ± 63	AB	514 ± 50	B	478 ± 11	B	692 ± 28	A	598 ± 70	AB	632 ± 61	A	↑
2-Methylbutanol	27720	25387 ± 627	B	26840 ± 252	AB	27397 ± 798	A	27080 ± 1057	AB	25777 ± 1487	A	26877 ± 676	A	25173 ± 862	A	27397 ± 1166	A	↓
i-Butanol	27450	27823 ± 1062	A	28537 ± 1074	A	26857 ± 955	A	24063 ± 425	B	26403 ± 2527	A	28240 ± 941	A	25647 ± 116	A	25253 ± 1116	A	↕
3-Methylbutanol	120050	121000 ± 2739	B	131253 ± 506	A	127977 ± 4119	AB	126523 ± 4243	AB	122353 ± 7008	A	126497 ± 2863	A	121387 ± 2470	A	128783 ± 6443	A	↑
2-Phenylethanol	36210	36273 ± 692	B	38870 ± 501	A	37077 ± 783	AB	37820 ± 1399	AB	38977 ± 1447	A	39450 ± 782	A	36090 ± 688	A	39237 ± 2142	A	↑
Totals	211970	182660		226108		219935		216001		213988		221756		208895		221302		↑
Esters																		
Acetic acid ethylester	85430	77800 ± 3162	C	113250 ± 2868	B	115540 ± 9616	B	137890 ± 7827	A	95417 ± 14245	B	97497 ± 8159	B	96950 ± 2869	B	127927 ± 7543	A	↑
Acetic acid phenylethylester	465	479 ± 7	A	424 ± 8	B	405 ± 27	BC	373 ± 7	C	392 ± 16	BC	442 ± 8	A	428 ± 25	B	367 ± 5	C	↓
Acetic acid 3-methylbutylester	1112	1149 ± 21	A	1032 ± 31	AB	1017 ± 82	B	830 ± 14	C	912 ± 13	BC	1070 ± 45	A	1024 ± 109	AB	792 ± 15	C	↓
Acetic acid 2-methylbutylester	90	74 ± 3	A	67 ± 4	B	72 ± 1	AB	70 ± 2	AB	61 ± 2	B	71 ± 4	A	67 ± 4	AB	65 ± 3	AB	↓
Acetic acid hexylester	126	119 ± 2	A	101 ± 7	B	103 ± 9	B	103 ± 1	B	94 ± 4	B	109 ± 7	A	105 ± 5	AB	97 ± 3	B	↓
Propionic acid ethylester	23	27 ± 0	B	31 ± 2	B	31 ± 2	B	41 ± 2	A	29 ± 1	B	29 ± 1	B	27 ± 1	B	37 ± 2	A	↑
i-Butyric acid ethylester	36	46 ± 2	B	44 ± 3	B	45 ± 1	B	54 ± 2	A	45 ± 1	AB	45 ± 1	B	40 ± 1	C	49 ± 2	A	↑
Butyric acid ethylester	184	203 ± 6	A	191 ± 2	A	202 ± 6	A	171 ± 4	B	187 ± 1	A	192 ± 2	A	190 ± 10	A	162 ± 3	B	↑
Lactic acid ethylester	11	166567 ± 7704	A	155693 ± 11440	A	166157 ± 3003	A	117607 ± 13970	B	148860 ± 23982	A	143163 ± 142	A	160103 ± 4580	A	134697 ± 5523	A	↑
Succinic acid diethylester	nq	283 ± 4	B	406 ± 9	A	298 ± 31	B	251 ± 18	B	613 ± 64	A	506 ± 26	A	262 ± 29	B	219 ± 45	B	↑
Hexanoic acid ethylester	410	433 ± 9	A	389 ± 24	A	419 ± 24	A	416 ± 15	A	390 ± 6	A	408 ± 11	A	394 ± 15	A	358 ± 15	B	↕
Octanoic acid ethylester	764	818 ± 60	A	697 ± 70	A	738 ± 99	A	707 ± 48	A	688 ± 15	B	726 ± 36	A	701 ± 54	B	722 ± 19	B	↓
Decanoic acid ethylester	306	294 ± 20	A	271 ± 17	A	264 ± 33	A	283 ± 28	A	279 ± 8	A	259 ± 29	A	261 ± 39	A	302 ± 6	A	↓
Totals	88957	248291		272595		285293		258797		247968		244518		260551		265795		↑
Acids																		
Hexanoic acid	5430	5723 ± 31	A	5913 ± 67	A	5937 ± 131	A	5680 ± 164	A	6037 ± 81	A	6047 ± 21	A	5890 ± 27	AB	5723 ± 176	B	↑
Octanoic acid	5850	5973 ± 55	A	5740 ± 72	A	5613 ± 424	A	5743 ± 140	A	5757 ± 71	B	5987 ± 49	A	5793 ± 84	B	5633 ± 76	B	↓
Decanoic acid	1520	1770 ± 20	A	1560 ± 78	B	1577 ± 55	AB	1590 ± 125	AB	1630 ± 44	A	1623 ± 29	AB	1507 ± 47	BC	1500 ± 60	C	↑
Totals	12800	13467 ±		13213 ±		13127 ±		13013 ±		13423 ±		13657 ±		13190 ±		12857 ±		↑
Terpenes																		
trans-Linalool oxide	21	26 ± 0	B	27 ± 0	B	27 ± 1	B	33 ± 2	A	29 ± 1	AB	28 ± 1	B	27 ± 0	B	32 ± 2	A	↑
cis-Linalool oxide	9	12 ± 0	B	11 ± 0	B	12 ± 0	B	17 ± 2	A	14 ± 0	AB	13 ± 1	B	12 ± 1	B	15 ± 1	A	↑
Linalool	23	27 ± 0	A	24 ± 1	B	26 ± 1	A	26 ± 0	A	25 ± 1	A	26 ± 0	A	25 ± 1	A	25 ± 1	A	↑
a-Terpineol	15	19 ± 1	B	18 ± 1	B	19 ± 0	B	23 ± 0	A	21 ± 1	AB	19 ± 0	B	19 ± 1	B	22 ± 2	A	↑
Totals	69	83		81		83		99		88		85		83		94		↑

Data are mean values of three experiments ± standard deviation. Different capital letters within the same row and within the same bacterial strain are significantly different according to the Tukey test ($p \leq 0.05$).

[a] General trend: ↑ all MLF strains increased ester concentration, ↓ ester concentration decreased, ↕ strain- and wine condition-specific differences.

Quantitatively, levels of short–chain esters such as lactic acid ethylester (milky notes, strawberry), succinic acid diethylester (fruity) and acetic acid ethylester (fruity) were the esters with the main concentration increases during MLF. The treatments with sequential inoculation had the lowest concentration of lactic acid ethylester and succinic acid diethylester, but the highest content of acetic acid ethylester. These compounds were also affected by the bacterial strain. Strain R1105 showed largest increase in lactic acid ethylester and acetic acid ethylester, while the wines inoculated with R1124 at 24h and 40 % of AF the highest content of succinic acid diethylester was detected.

The content of the fruity ethylesters propionic acid ethylester, i-butyric acid ethylester and butyric acid ethylester increased significantly in all wines after MLF. In the wines with sequential MLF the highest concentration of propionic acid ethylester and i-butyric acid ethylester was noted while the butyric acid ethylester showed the lowest increase compared to the wines with simultaneous inoculation. In addition, bacterial strain differences were observed. *O. oeni* R1105 tended to produce higher concentrations of ethyl esters (Figure 5).

A decrease of the longer chained esters, such as hexanoic-, octanoic- and decanoic acid ethylester, was observed in most wines after MLF. An higher content of hexanoic acid ethylester (green apple) was noted in the wines fermented with R1105.

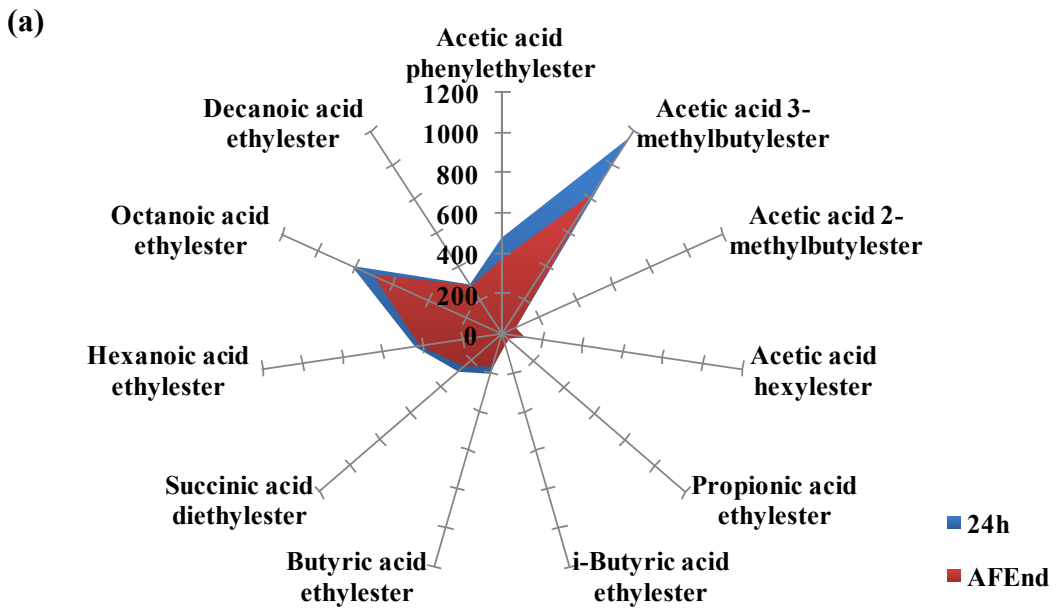

Figure 5 a. Changes in the esters profiles (µg L^{-1}) associated with MLF by bacterial starter culture *O. oeni* R1105 in the co-inoculation (24h) and sequential MLF (AFEnd) treatments.

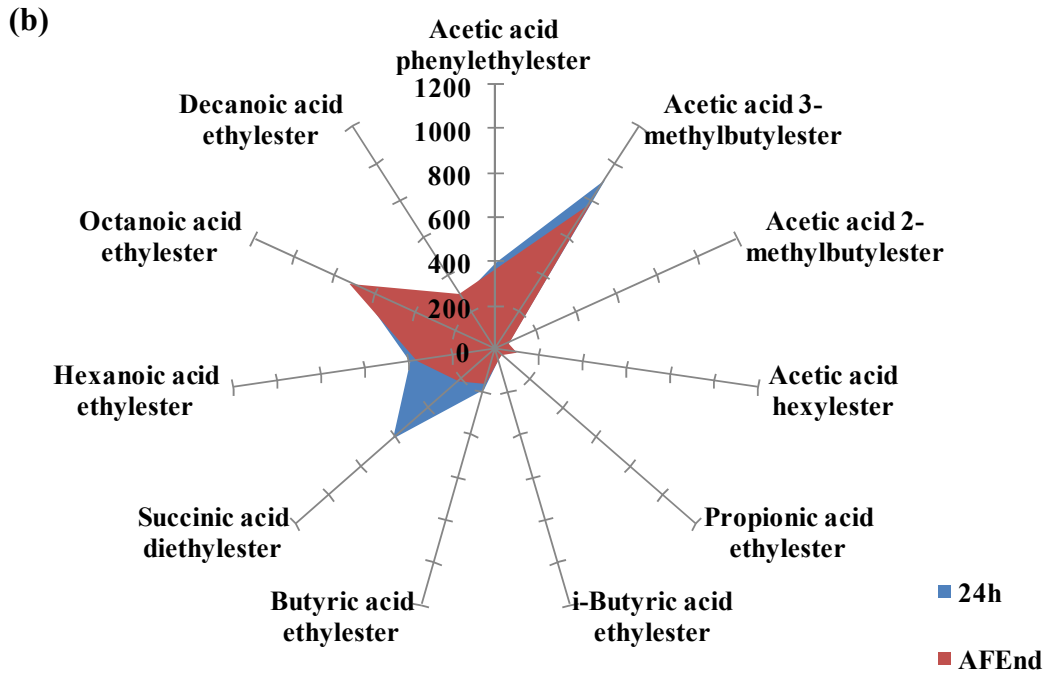

Figure 5 b. Changes in the esters profiles (µg L^{-1}) associated with MLF by bacterial starter culture *O. oeni* R1124 in the co-inoculation (24h) and sequential MLF (AFEnd) treatments.

Figure 6 illustrates the concentration of total ethyl esters (without lactic acid ethylester) and the concentration of total acetate esters (without acetic acid ethylester) in the wines after MLF.

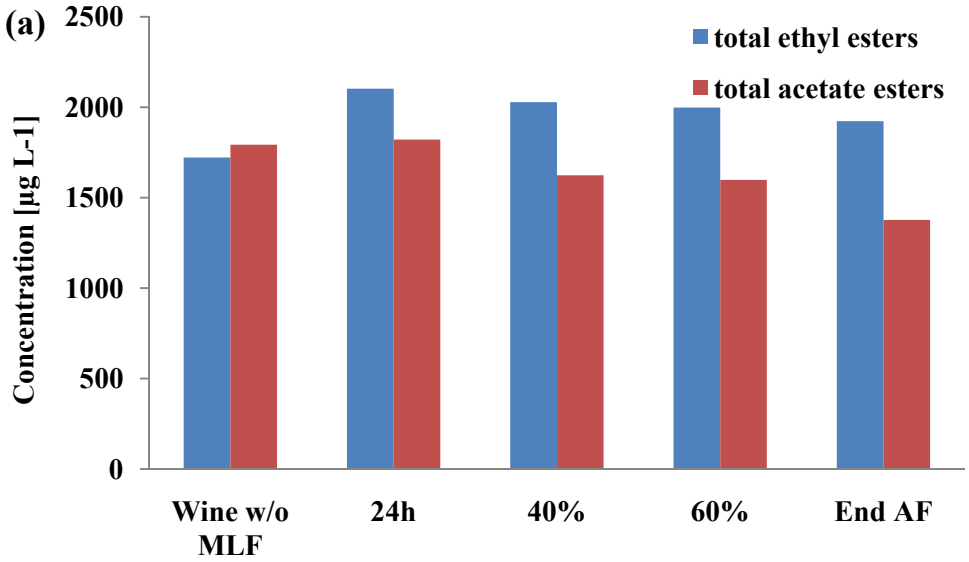

Figure 6 a. Average concentrations of total ethyl esters (without lactic acid ethylester) and the concentration of total acetate esters (without acetic acid ethylester) at the end of MLF in wines inoculated with *O. oeni* R1105.

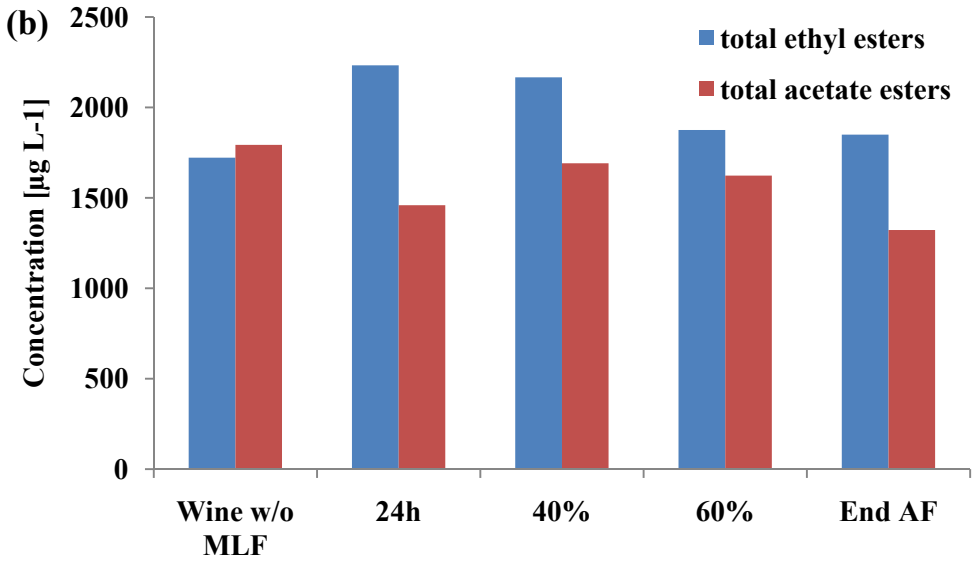

Figure 6 b. Average concentrations of total ethyl esters (without lactic acid ethylester) and the concentration of total acetate esters (without acetic acid ethylester) at the end of MLF in wines inoculated with *O. oeni* R1124.

Acids

Volatile fatty acids such as hexanoic and decanoic acid, generally increased in all the wines once MLF had finished while the content of octanoic acid decreased. The lowest concentration of hexanoic acid was found in the wines with sequential inoculation.

Terpenols

A raise of the concentration of the terpenols trans-linalool oxide, cis-linalool oxide, linalool and α-terpineol was observed in all wines after MLF. The wines with sequential MLF had the highest content of trans-linalool oxide, cis-linalool oxide and α-terpineol and total terpenols (Figure 7).

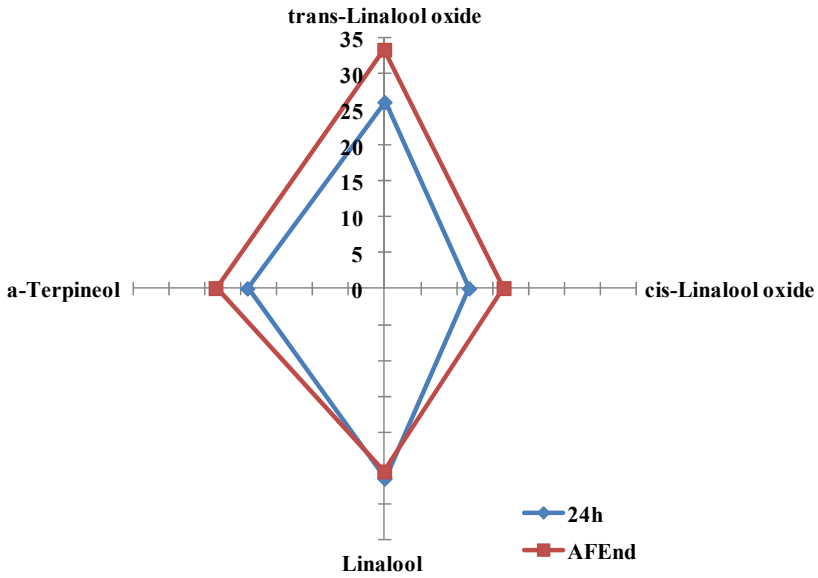

Figure 7. Changes in the terpenols profiles (µg L^{-1}) associated with MLF by *O. oeni* R1105 in the co-inoculation (24h) and sequential MLF (AFEnd) treatments. Similar data was obtained for *O. oeni* R1124 (Table 3).

Multivariate data analysis

As an overview of the results, a principal component analysis (PCA) of the volatile aroma compounds of the wines was performed. 81 % of the variance was explained by the first two principal components. As shown in Figure 8, these PCA's separated the samples according to inoculation time. Moreover, the treatments inoculated for MLF at 24h and 40 % of AF could be further separated according to the bacterial strain used. Also the wine without MLF is clearly distinguishable from the wines with MLF. On the score plot separation along PC1 was associated with discrimination of treatments inoculated at 40 % of AF and at 24h with R1124 from the treatments inoculated at 60 % of AF, at the end of AF and at 24h with R1105. Loadings for succinic acid diethylester were correlated with treatments inoculated at 40 % of AF and at 24h with R1124, while loadings for acetic acid 3-methylbutylester were correlated with the inoculation at 24 h with R1105.

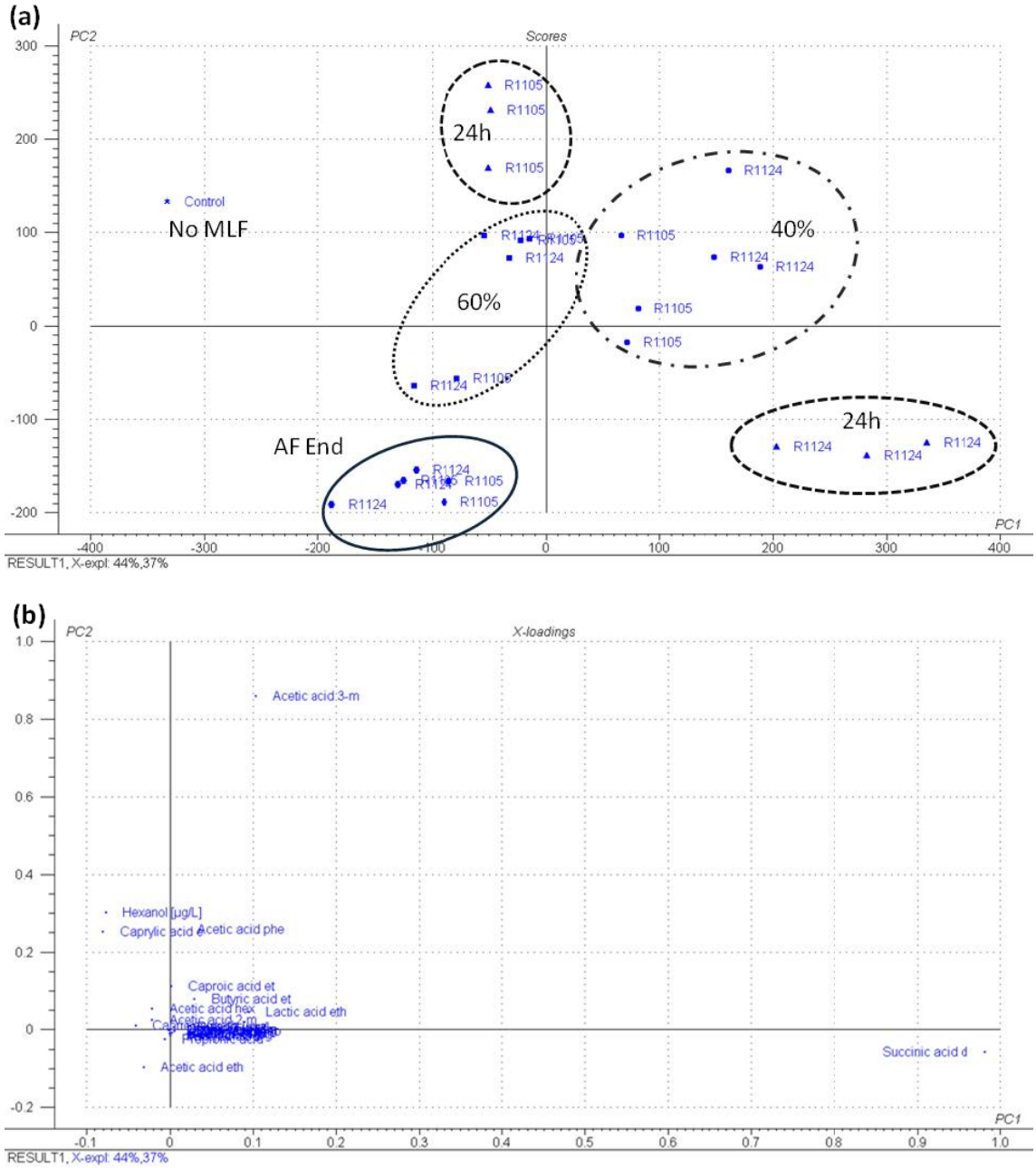

Figure 8. PCA score plot derived from volatile aroma compounds of all Riesling wines following MLF and the control wine at the end of AF without MLF (no MLF). (a) Scores plot and (b) the corresponding loadings plot.

Discussion

Successful MLF in cool climate Riesling wines is often difficult to achieve. This study has verified the feasibility of simultaneous inoculation in low pH wines with two suitable yeast-bacterium combinations. The results shown here point out that it is possible to inoculate the bacterial culture at different timings of AF without, on the one hand, inhibiting AF or, on the other hand, causing failure of MLF. However, pH values and timing of bacterial inoculation were shown to be important for how rapidly MLF commences. These results are in agreement with a previous study (Rosi et al. 2003), carried out in a commercial white grape juice, reporting the possible inoculation with LAB at the beginning, middle, and end of AF without slowing down or inhibiting AF or causing failure of MLF. Yet, at pH 3.2 a lowering of bacterial viability was observed (Rosi et al. 2003). In the present study, the co-inoculation at 40 % of AF seemed to be the inhibitoriest time for malolactic bacteria to start MLF. At this time the pH of the medium was 2.9 which can be associated with production of acids by yeast metabolism. These findings were also noted by Rosi et al. (2003), when bacteria were inoculated halfway through AF. On the other hand, when MLF was carried out at the end of AF, the ethanol content displayed an additional inhibiting factor which delayed the beginning of MLF. Generally, a reduction in total fermentation time was observed when using simultaneous inoculation techniques compared to traditional sequential MLF. The time gained was ranging between 25 and 50 days, depending on the bacterial strain and inoculation time used. This represents an important advantage for the wineries regarding the process efficiency. However, when *O. oeni* R1124 was used, the length of MLF itself in the treatments with simultaneous inoculation was similar or longer than their respective sequential treatment. *O. oeni* R1105, on the other hand, carried out MLF faster in the simultaneous treatments than in the consecutive ones and was generally less inhibited by the low pH than R1124. *O. oeni* R1124 seems to be better suited for a sequential MLF, while strain R1105 can be used for both, co-inoculation and sequential MLF. The same strain tendencies were observed in other studies done in red wine (personal communication Krieger-Weber, 2011).

The benefits and risks of sequential and simultaneous AF / MLF remain controversial. In this study no negative effect on final wine quality could be substantiated. It was sug-

gested that simultaneous inoculation of yeast and bacteria could result in increased concentration of acetic acid produced by LAB in the presence of available sugars in the must (Davis et al. 1985). In the co-inoculation treatments, levels of acetic acid never exceeded 0.7 g L^{-1}, considering that the wines without bacterial inoculation contained levels between 0.5 and 0.8 g L^{-1}. It can be assumed that in none of the simultaneous fermentations the bacteria produced worrisome levels of acetic acid from sugar and that the yeast metabolism contributed to the elevated acetic acid levels. Thus confirming results of other studies (Semon et al. 2001; Jussier et al. 2006; Massera et al. 2009), demonstrating the possibility of simultaneous fermentation without excessive increase of volatile acidity. Little is known about the impact of simultaneous inoculation on the production of volatile aroma compounds. All acetate esters, except acetic acid ethylester, decreased following MLF, while the ethyl esters increased. This is in accordance with previous studies on sequential MLF in red wines (Ugliano and Moio 2005; Bartowsky et al. 2008). Based on sensorial data, it was suggested that Chardonnay, Malbec and Shiraz wines fermented with co-inoculation tend to be fruitier than the wines with sequential inoculation (Bartowsky et al. 2002; Jussier et al. 2006; Massera et al. 2009). In this study the Riesling wines with sequential MLF had the lowest concentration of acetate esters and ethyl esters, most notably due to lower concentrations of acetic acid phenylethylester, acetic acid 3-methylbutylester, butyric acid ethylester, lactic acid ethylester and succinic acid diethylester. This might potentially result in decreased fruitiness in wines with consecutive MLF. The wines with the 24 h inoculation, on the other hand, had the highest concentration of fruity ethyl esters. In addition, changes in the ester concentrations were also affected by the bacterial strain used. *O. oeni* R1105 seemed to produce higher concentrations of various fruity esters, such as propionic acid ethylester, butyric acid ethylester or lactic acid ethylester, associated with fruitiness, milky notes and mouthfeel, respectively. Increased concentration of these or other esters and strain dependency was also observed by others (Maicas et al. 1999; Pozo-Bayón et al. 2005; Ugliano and Moio 2005; Boido et al. 2009). Comparison of fermentation-derived compounds from treatments with simultaneous and sequentially inoculated MLF, has illustrated that the profiles of the wines produced, were very different as a result of the MLF inoculation regime and *O. oeni* strain. The profiles of fermentation-derived compounds of the wines that conducted MLF are clearly distinguishable from those that did not. In addition, wines with complete MLF could be clearly separated

according to inoculation timing and distinguished from the wine without MLF. Also, the treatments inoculated for MLF at 24 h and 40 % of AF could be further separated according to bacterial strain used.

In conclusion, to our knowledge, for the first time the impact of different bacterial inoculation timings on the MLF performance and on the production of volatile aroma compounds in low pH Riesling must was accomplished. No negative impact of simultaneous AF / MLF on the fermentation success and on the final wine quality was observed. It was demonstrated that inoculation timing and the bacterial strain used can affect the outcome of the final volatile aroma composition of the wine. Applying a co-inoculation protocol may offer microbiological, technological and sensorial advantages, especially in low-pH, cool-climate white musts with potential high alcohol content. However, the success of simultaneous vinification will also depend on the selection of suitable yeast-bacterium combinations (Alexandre et al. 2004).

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2.3 Cloning and characterisation of a cystathionine β/γ-lyase from two *Oenococcus oeni* oenological strains

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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Cloning and characterisation of a cystathionine β/γ-lyase from two *Oenococcus oeni* oenological strains

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Abstract Sulphur-containing compounds in wine have been extensively studied because of their effect on wine flavour and quality. In this study, an enzyme that degrades sulphur-containing amino acids was cloned and characterised from two *Oenococcus oeni* strains of oenological origins. The enzyme has features of a cystathionine-γ-lyase (EC 4.4.1.1), a pyridoxal-5-phosphate-dependent enzyme catalysing an α,γ-elimination reaction of L-cystathionine to produce L-cysteine, α-ketobutyrate and ammonia. Moreover, it was able to catalyse an α,β-elimination reaction producing homocysteine, pyruvate and ammonia from L-cystathionine. An elimination reaction of L-cysteine and DL-homocysteine was also efficiently catalysed by the enzyme, resulting in the formation of hydrogen sulphide. Furthermore, the ability to demethiolate methionine into methanethiol, an unfavourable volatile sulphur compound in terms of wine aroma, was observed. The findings of this work suggest that *O. oeni* seems to play a minor role in the production of volatile sulphur compounds during the vinification process as the optimal conditions were far from the harsh wine environment.

Keywords *Oenococcus oeni* · Cystathionine lyase · Volatile sulphur compounds · Enzyme activity

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Introduction

Although hundreds of chemical compounds have been identified in grapes and wines, only a few actually contribute to the sensory perception of wine aroma and flavour (Polásková et al. 2008). Formation of aroma in wine is a complex process and mainly carried out by the microflora present during alcoholic fermentation and malolactic fermentation (MLF). The metabolic activity, as well as the kinetics of both fermentations, will influence the sensory profile of the wine depending on the vinification techniques and the physical and chemical composition of the wine. Besides the primary function of lactic acid bacteria (LAB) to conduct MLF, they can also alter the wine composition (Liu 2002); however, these activities as well as the sensory impact of LAB are less well understood and poorly characterised when compared to wine yeast. Swiegers et al. (2005) listed the possible pathways by which LAB are able to produce volatile compounds by, e.g. metabolising grape components or modifying the yeast derived secondary metabolites. Although the genome of

Oenococcus oeni has been sequenced and analysed (Mills et al. 2005), there is limited information on the genes in this bacterium or their potential contribution to wine aroma. The only genes which have been cloned and characterised are *alsD* and *alsS* (Garmyn et al. 1996) which are involved in the diacetyl synthesis, conferring "buttery" aroma and flavour in wine (Bartowsky and Henschke 2004). Recently, Sumby et al. (2009) reported on the cloning and characterisation of *EstB*28, the first esterase from *O. oeni* to be characterised with the potential to alter the ester profile of wine.

Among the wine substrates catabolised by LAB, amino acids represent the most important source of nitrogen, carbon and sulphur for sulphurous amino acids (Pripis-Nicolau et al. 2004). Extensive research has been carried out on the enzymes responsible for volatile sulphur compounds (VSC) produced in dairy-associated LAB (Bruinenberg et al. 1997; Seefeldt and Weimer 2000; Yvon and Rijnen 2001; Fernandez et al. 2002; Gente et al. 2007; Irmler et al. 2008; Hanniffy et al. 2009) while VSC production by wine-related LAB is not well documented. VSC can also contribute to the wine aroma, with some compounds being classified as detrimental to wine quality, depending on their concentration; other compounds can contribute positively to the bouquet of wine (Mestres et al. 2000; Landaud et al. 2008). Pripis-Nicolau et al. (2004) demonstrated that strains of *O. oeni* converted methionine to diverse VSC. One of these, 3-(methylthio)-propionic acid, is characterised by chocolate and roasted odours and mainly contribute to the aroma of red wines. The production of methionol by different wine LAB has also been reported (Henick-Kling 1995; Ugliano and Moio 2005). The pathways leading to VSC biosynthesis are only partially studied in *O. oeni*, whereas they are well-known in LAB and, more importantly, in cheese microflora, in which VSC are important cheese-flavour components. Vallet et al. (2008) studied pathways that produce VSC and showed that methional and 2-oxo-4-(methylthio)-butyric acid are key intermediates for VSC synthesis from methionine in *O. oeni*; however, the metabolic pathway in *O. oeni* for the formation of these compounds is not yet clarified. The conversion of sulphur-containing amino acids into VSC proceeds via two distinct routes, transamination and elimination (Yvon and Rijnen 2001; van Kranenburg et al. 2002; Ardo 2006). The first pathway goes through α-keto acid intermediates and is mainly initiated by a transamination reaction catalysed by aminotransferases. The second one is initiated by elimination reaction catalysed by lyases which cleave the side chain of amino acids. The possible involvement of lyases in VSC biosynthesis has been mainly studied in cheese. The activity of cystathionine β-lyase (CBL) and cystathionine γ-lyase (CGL), pyridoxal-5′-phosphate (PLP)-dependent enzymes, has been detected in several LAB such as *Lactococcus lactis* (Alting et al. 1995), *Lactobacillus fermentum* (Smacchi and Gobbetti 1998), *Lactobacillus helveticus* and *Lactobacillus casei* (Dias and Weimer 1998; Irmler et al. 2008). Moreover, these enzymes have been purified and characterised (Bruinenberg et al. 1997; Smacchi and Gobbetti 1998; Gente et al. 2007; Irmler et al. 2008). The CBL converts L-cystathionine to L-homocysteine, pyruvate and ammonia via α,β-elimination reaction. The CGL converts L-cystathionine to L-cysteine, α-ketobutyrate and ammonia via α,γ-elimination reaction. Although their main function is anabolic, they also possess α,β- and α,γ-elimination activities (Landaud et al. 2008). Moreover, it was shown that the enzymes are capable of converting other sulphur-containing substrates via α,γ-elimination reaction (Weimer et al. 1999; Yvon and Rijnen 2001).

To more completely characterise lyases of wine-associated LAB, the enzymes and their structural genes must be fully investigated. This paper describes the identification, heterologous expression and biochemical characterisation of a cystathionine lyase from two *O. oeni* strains. These enzymes have the ability to carry out both the α,β-elimination and α,γ-elimination reactions on sulphur-containing amino acids such as cystathionine, cysteine, homocysteine and methionine.

Materials and methods

Strains, media and growth conditions

Two commercial *O. oeni* strains (R1105, R1106) (Lallemand, Toulouse, France) were used in this study. Both strains have been deposited in the German Collection of Microorganisms and Cell Cultures under the following numbers: DSM14493 (R1105) and DSM14511 (R1106).

The strains were grown in modified MRS (Roth, Karlsruhe, Germany) supplemented with 20% pasteurised apple juice (Geisenheim Research Center, Germany) and the pH was adjusted to 5.2. The LAB strains were cultured at 30 °C. *Escherichia coli* strains BL21(DE3) and DH5-α were grown in Luria-Bertani (LB) broth at 37 °C on a rotary wheel at 220 rpm. Selective antibiotic concentration was 100 μg/mL of ampicillin (Sigma Aldrich, Steinheim, Germany) when required.

DNA isolation and manipulations

Total *O. oeni* genomic DNA was isolated as described by Lewington et al. (1987). Plasmid DNA from *E. coli* DH5-α was extracted with the GeneJET™ Plasmid Miniprep Kit (Fermentas GmbH, St. Leon-Rot, Germany) according to the manufacturer's recommendations. All cloning steps were conducted according to standard procedures as described in Sambrook and Russell (2001). T4 DNA ligase and all

restriction enzymes were purchased from Roche (Mannheim, Germany). Primers for the polymerase chain reaction (PCR) were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA). PCR was performed with Ex Taq polymerase (TaKaRa, Bio Inc., Shiga, Japan) in a TRIO-Thermoblock (Biometra, Göttingen, Germany) according to the parameters specified. PCR resulting fragments were separated by 1% agarose gel electrophoresis, and when required, purified with ™Gel Extraction Kit (Fermentas GmbH) according to the protocol supplied by the manufacturer.

Cloning and heterologous expression of *cgl*

Both *O. oeni* strains were tested for the presence of the cystathionine gamma lyase (*cgl*) gene (OEOE_1758/EC:4.4.1.1) by PCR amplification. Primers were designed based on the *O. oeni* PSU-1 genome sequence (GenBank accession no. CP000411.1). Purified genomic DNA served as template in the PCR. To allow subsequent cloning, *Nde*I and *Bam*HI sites were introduced at the 5′ and 3′ ends of the primers (designed in this study): 5′-GTCCATATGATGAAATTCAATACAAAACTTATTCATG-3′ and 5′-ATCGGATCCCTAAATCTTGCTGAATGAC-3′ (restriction sites are underlined). Amplification included denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and polymerization at 72 °C for 1.20 min. A final elongation step was performed at 72 °C for 5 min.

Plasmids pET14.b::cgl05 and pET14.b::cgl06 were constructed by inserting the amplified PCR products from *O. oeni* R1105 and R1106 into the *Nde*I and *Bam*HI restriction enzyme sites of pET14b (Studier et al. 1990). *E. coli* DH5-α was used as a host strain for cloning and maintenance of the plasmids. *E. coli* BL21(DE3) was used for the expression of the *cgl* gene under the control of an inducible T7 promoter.

DNA sequencing

The amplified products were cloned into the pGEM-T Easy (Promega, Cape Town, South Africa) vector and transformed into *E. coli* DH5-α. DNA sequencing was performed by the Central Analytical Facility of Stellenbosch University, South Africa. DNA sequences were obtained by performing cycle sequencing reactions using the Applied Biosystems BigDye Terminator Ready Reaction Kit v3.1, following the manufacturer's instructions. Samples were run on an Applied Biosystems 3130xl Genetic Analyzer and analysed using a Sequencing Analysis 5.2 software.

Expression and purification of His-tagged proteins

E. coli BL21(DE3) harbouring the pET14.b::cgl05 and pET14.b::cgl06 plasmids were grown in 200 mL of LB broth supplemented with the required antibiotic to an OD_{600nm} of 0.6 at 37 °C at 220 rpm. Induction of genes was performed under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter by adding 0.4 mM of IPTG. The cultures were grown for another 4 h at 30 °C at 220 rpm. The *E. coli* cells were harvested (5,000 rpm, 4 °C, 15 min), washed twice with 0.2 M sodium phosphate buffer (pH 7.4) and frozen at −20 °C. The frozen pellet was resuspended in 4 mL of binding buffer (20 mM sodium phosphate (pH 7.4), 150 mM NaCl, 20 mM imidazole) and approximately 0.2 g of glass beads (212–300 μm) was added. Vigorous agitation in a Mini-BeadBeater-8 (Biospec Products, Inc.) was used for the disruption of the cells. The extract was cleared by centrifugation (5,000 rpm, 4 °C, 10 min) and then applied to a 1-mL HiTrap Chelating HP column (GE Healthcare, Uppsala, Sweden) which had been loaded with 0.5 mL 0.1 M $NiSO_4$ and equilibrated with binding buffer. The column was then washed with binding buffer, and bound proteins were subsequently eluted with 20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl and 500 mM imidazole. Removal of the imidazole was done by applying the eluate to NAP columns (GE Healthcare) which had been equilibrated with 20 mM sodium phosphate (pH 7.4) beforehand according to the manufacturer's instructions. The eluted protein fractions were analysed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualised by colloidal Coomassie brilliant blue staining.

The protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany) based on the method of Bradford (1976), with bovine serum albumin as the standard.

Determination of enzyme activity

The standard reaction mixture of 200 μL consisted of 50 mM sodium phosphate (pH 6.8 or 5.5), 5 μM PLP, various concentrations of substrate (0.1–25 mM final concentration) and enzyme solution (5 μg/200 μL). The reactions were performed at 37 °C. Following substrates were tested: L-cystathionine, L-cysteine, DL-homocysteine and L-methionine. The release of thiols from cystathionine and methionine was determined by adding 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Uren (1987). A yellow colouration developed when thiols were released and was recorded at 3-min intervals at 412 nm for 60 min or every 10 min for 3 h. Production of α-keto acids was determined with 3-Methyl-2-benzothiazolinone as described by Esaki et al. (1987). α-Keto acid identification was carried out by high-performance liquid chromatography (HPLC). Therefore, assays were stopped after 40 min of incubation by the addition of 2 μL of H_2SO_4 (380 mM) and by heating at 95 °C for 5 min. The release of volatile sulphur

compounds was measured by gas chromatography-pulsed flame photometric detection (GC-PFPD) as described below. The Hanes–Woolf transformation (S/V vs. $1/S$), where V is the formation rate of either thiols or keto acids (micromol per minute per milligramme protein) and S is the concentration (millimolar) of each substrate, was used to calculate K_m and V_{max} values. All assays were performed in triplicate.

To assay cystathionine synthase activity, the purified enzyme was incubated with *O*-succinyl-*L*-homoserine in the presence of L-cysteine. The formation of acids from the substrates was analysed by HPLC.

Influence of temperature, pH and ethanol on enzyme activity

The influence of temperature was studied by pre-incubating the purified enzyme for 30 min at five temperatures across the range of 20 to 75 °C. The effect of pH on enzyme activity was determined by varying the pH of the reaction mixture across the range pH 3.0 to 9.0 using 0.1 M sodium phosphate buffer. Furthermore, the impact of the presence of ethanol at four concentrations from 5% to 15% (*v/v*) was investigated. Enzyme activities were determined as described above.

HPLC analysis

Separation of the filtrate was performed on an Aminex HPX-87H (300 by 7.8 mm; Bio-Rad) protected with a SecurityGuard™ cartridge system (Phenomenex). The operating conditions were as follows: a flow rate of 0.5 mL/min, 46 °C and detection at 210 nm. The mobile phase was 3.8 mM H_2SO_4. Pyruvate and α-ketobutyrate (Sigma-Aldrich) were used as standards.

Gas chromatography-pulsed flame photometric detection

Detection of VSC was carried out by GC-PFPD analysis. The enzymatic assays were done as described above in a 1-mL final volume. When methionine was used as a substrate, the reaction mixture was incubated for 6 h instead of 40 min. Sample preparation and analysis of VSC were performed as described previously (Rauhut et al. 2005; Irmler et al. 2008).

Results

DNA sequencing and sequence analysis

The two *O. oeni* strains were tested for the presence of the *cgl* gene (OEOE_1758) and were successfully amplified from both strains by PCR. The amplified PCR product consisted of 1,140 nucleotides encoding a deduced protein of 379 amino acids. The sequences were aligned with the *O. oeni* strain PSU-1 genome sequence (GenBank accession no. CP000411.1), and analysis showed that the sequences were highly homologous to PSU-1. Four nucleotides in R1105 and six nucleotides in R1106 compared to PSU-1 (Online Source 1) were different leading to two respectively four amino acid substitutions. The deduced sequence of R1105 showed amino acid substitutions at position 184 and 291. Leucine was replaced with phenylalanine and cysteine with arginine. For strain R1106 the alanine at position 121 and the serine at position 328 were exchanged to threonine and glycine, respectively. In addition, strain R1106 had the same amino acid substitutions as R1105 at the positions 184 and 291.

Expression of *cgl* into *E. coli*

The *cgl* genes from R1105 and R1106 were cloned into pET14b. The *E. coli* BL21 (DE3) transformants containing either the pET14.b::cgl05 or the pET14.b::cgl06 plasmid were induced and soluble recombinant His-tagged proteins named Cgl05 and Cgl06 were obtained. Proteins were then purified under native conditions by affinity chromatography. Enzyme purification was monitored by analysing aliquots of protein fractions from each chromatography step by SDS-PAGE. A single polypeptide band of approximately 40 kDa (predicted 41.5 kDa) was observed in the final enzyme preparation (Fig. 1). The purified enzymes were then used for further biochemical characterisation.

Determination and characterisation of enzyme activity

Substrate specificity of both recombinant CGLs, Cgl05 and Cgl06, were determined using various substrates by assaying the formation of the products. Both showed high activity towards the degradation of L-cystathionine. They also degraded all other substrates tested. Table 1 shows the comparison of recombinant CGL isolate from *O. oeni* R1105 (CGL05) and R1106 (CGL06). There were no statistical significant differences in the kinetic parameters between the two strains. DTNB was added to measure the release of thiols from cystathionine and methionine. Figure 2 shows the thiol production of the enzyme using L-methionine and L-cystathionine at various substrate concentrations. The values of the controls (assays without enzyme) were subtracted from the values of the assays with enzyme. Using methionine as substrate, we observed an enzyme-catalysed cleavage to free thiols; however, we were not able to saturate the reaction with methionine. For this reason, V_{max} and K_m could not be determined.

By using L-cystathionine as substrate, the activity of the purified enzymes was highest at pH 8.0. No activity was observed at a pH below 6.5. In contrast, L-methionine was degraded at pH 5.5 and 6 (Online Source 2).

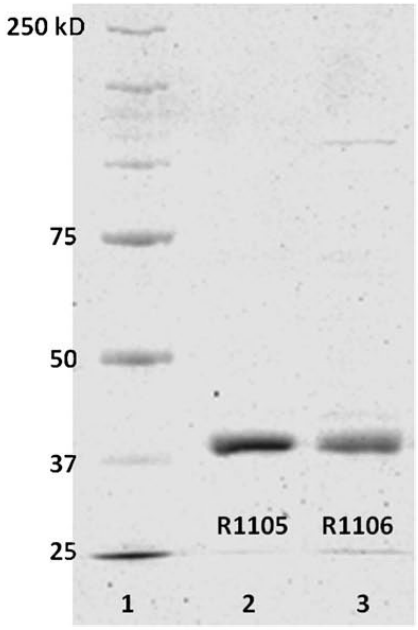

Fig. 1 SDS-PAGE analysis of purified Cgl proteins. *Lanes 1*, molecular mass marker proteins (in kilodaltons); *2*, Cgl05 from pCGL R1105; *3*, Cgl06 from pCGL R1106

To clarify whether an α,β- or an α,γ-elimination took place, products released during the enzyme reactions were analysed. First, α-keto acids were determined by HPLC. The results showed that cystathionine was degraded into pyruvate and α-ketobutyrate. Furthermore, it was found that pyruvate and α-ketobutyrate were formed from L-cysteine and DL-homocysteine, respectively (Fig. 3). Assays performed with each substrate in absence of the enzyme served as controls. Chemical degradation to organic acids was not detected (data not shown). Second, it was observed that reactions containing L-cysteine, DL-homocysteine or L-cystathionine as a substrate released a strong hydrogen sulphide scent (rotten egg odour) after incubation. This was confirmed by GC-PFPD (Fig. 4). Finally, the formation of ammonia was confirmed by using an enzymatic analysis kit (Boehringer, Mannheim, Germany) (data not shown).

To study whether the enzyme also exhibits cystathionine synthase activity, CGL05 and CGL06 were incubated with *O*-succinyl-*L*-homoserine in the presence and absence of *L*-cysteine. HPLC analysis showed that the CGL proteins formed α-ketobutyrate and succinate from *O*-succinyl-*L*-homoserine. Incubation of *O*-succinyl-*L*-homoserine together with L-cysteine resulted in the formation of pyruvate, α-ketobutyrate and succinate (Fig. 3). Serine was studied as a putative substrate, but it was not degraded. In order to confirm the release of methanethiol from methionine, GC-PFPD was utilised. Very low levels or no methanethiol, dimethyl disulphide and dimethyl trisulphide (DMTS) were detected in the headspace of the sample with methionine in the absence of enzyme, whereas the addition of enzyme, resulted in a release of methanethiol, dimethyl disulfide (DMDS) and DMTS (Fig. 4).

The effect of temperature and ethanol on enzyme activity was determined by using L-cystathionine as a substrate (Online source 3 and 4). The enzyme was stable up to 56 °C and was not inhibited by 15% (*v*/*v*) ethanol. After several weeks of storage at 4 °C or −20 °C, both enzyme preparations still showed cystathionine lyase activity.

Discussion

This research focused on the cloning and biochemical characterisation of a cystathionine lyase from two oenological *O. oeni* strains.

Only a few studies have been conducted on the amino acid catabolism of wine LAB, with the exception of arginine (Liu and Pilone 1998); however, the catabolism of amino acids by wine LAB is expected to have an impact on wine quality, given that a range of compounds can be produced, such as aldehydes, alcohols and acids, in addition to amines. Over 20 LAB genomes have been fully sequenced (Kleerebezem et al. 2003; Liu et al. 2005; Mills et al. 2005; Claesson et al. 2006; Mayo et al. 2008). The available genomic information provides additional possibilities to study the flavour-forming potential of LAB; however, biochemical methods are necessary to investigate enzymatic function, substrate specificity and activity of the genes of interest. Since methionine and cysteine are generally present in only limited quantities in wine, the formation of VSCs will depend on both biosynthesis and catabolic pathways of methionine and cysteine; however the metabolism of sulphur-containing amino acids is

Table 1 Kinetic parameters of the recombinant Cgl05 and Cgl06. Enzymatic assays were performed with sodium phosphate buffer (pH 6.8) at 37 °C

	CGL05		CGL06	
	K_m (mM)	V_{max} (μmol/min/mg)	K_m (mM)	V_{max} (μmol/min/mg)
L-Cysthationine	0.236±0.055	1.754±0.426	0.232±0.048	1.951±0.281
L-Cysteine	0.069±0.072	605.111±518.737	0.029±0.025	237.984±135.608
DL-Homocysteine	1.417±1.043	236.159±91.307	1.153±0.591	111.859±78.078

Data are means ± SD from three experiments

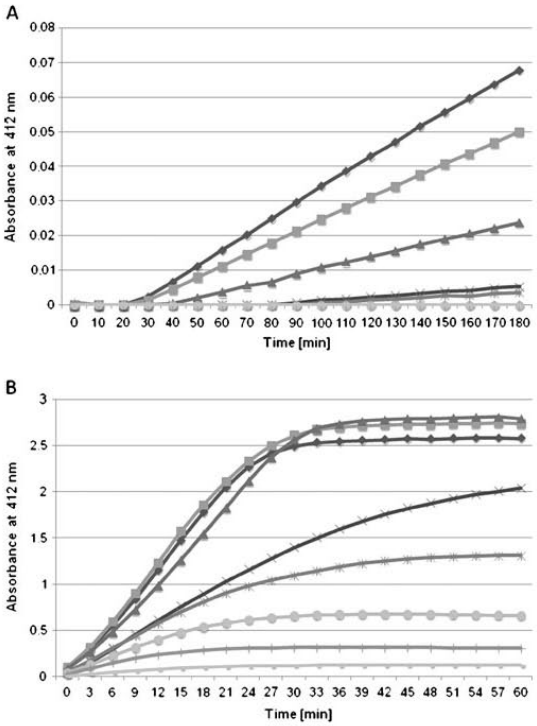

Fig. 2 Thiol production curves in the presence of L-methionine (**a**) and L-cystathionine (**b**) by incubating the recombinant Cgl05. Illustrated are sample values less the control values. Similar results were obtained with Cgl06 (data not shown). **a** The final substrate concentrations were 20 mM (*diamond*), 10 mM (*square*), 5 mM (*triangle*), 2 mM (*multiplication sign*), 1.5 mM (*asterisk*), 1 mM (*circle*). **b** The final substrate concentrations were 2 mM (*diamond*), 1 mM (*square*), 0.5 mM (*triangle*), 0.2 mM (*multiplication sign*), 0.15 mM (*asterisk*), 0.1 mM (*circle*), 0.05 mM (*plus sign*) and 0.02 mM (*minus sign*). Experiments were carried out in triplicate

diverse, especially considering the existence of multiple alternative pathways as well as several possible chemical reactions which also can contribute to VSC formation.

In this study, it was shown that *O. oeni* possesses a gene encoding a cystathionine lyase which is highly conserved among the three compared *O. oeni* strains. Applying comparative sequence analysis, Liu et al. (2008) revealed that genes encoding cystathionine lyases fall into two distinct families named CBL/CGL and CBL, which share little sequence similarity. The in silico translation of *cgl* of *O. oeni* was compared with other known and predicted CBL/CGL sequences using NCBI protein blast. Besides being nearly identical with the CGL (OEOE 1758) of *O. oeni* PSU-1, it showed 73% identity with the CBL/CGL of *Lactobacillus salivarius* UCC118 and ATCC 11741; 72% with the CGL of *Lactobacillus reuteri* DSM 20016 and 70% with *Lactobacillus fermentum* ATCC 14931. Furthermore, it showed 67% identity with Ctl1 of *Lactobacillus casei* FAM18168 67% with Ctl2 of *Lactobacillus casei* FAM18108, 60% with MetC of *Lactococcus lactis* subsp. *cremoris* SK11 and 55% with YrhB of *Bacillus subtilis* str. 168. The latter four genes also belong to the CBL/CGL family and have experimentally been shown to encode a cystathionine lyase exhibiting dual CBL/CGL activity. Probably all enzymes belonging to "CBL/CGL" may display a mixture of cystathionine β- and γ-lyase activities. It implies that LAB enzymes in this subcluster could have either solo CGL activity or a dual CBL/CGL activity (Liu et al. 2005). In this study, the gene product of *cgl* also showed dual CBL/CGL activity and formed pyruvate, α-ketobutyrate and hydrogen sulphide from cystathionine, released pyruvate and hydrogen sulphide from cysteine, and generated methanethiol from methionine. These observations suggest that cystathionine can be broken down in two possible steps. First, α-ketobutyrate, pyruvate, cysteine, homocysteine and ammonia are formed. Second, cysteine and homocysteine are further metabolised to pyruvate, α-ketobutyrate, ammonia and hydrogen sulphide. Interestingly, Cgl05 and Cgl06 also formed α-ketobutyrate and succinate from *O*-succinyl-*L*-homoserine. A combination of cysteine and *O*-succinyl-*L*-homoserine resulted in the additional release of pyruvate; however, compared with *O*-succinyl-*L*-homoserine as the sole substrate, the peak area of α-ketobutyrate was significantly reduced. This implies that the compound is used for cystathionine biosynthesis, and we think that the enzyme exhibits also cystathionine synthase activity.

These results lead to the conclusion that Cgl05 and Cgl06 are multifunctional PLP-dependent enzymes that on the one hand degrade cystathionine by an α,β- and an α,γ-elimination reaction and on the other hand exhibit cystathionine γ-synthase activity when *O*-succinyl-*L*-homoserine and L-cysteine are present. This attribute has also been reported for *MetB* of *Lactobacillus casei* (Irmler et al. 2008) and was found for Ctl1 (S. Irmler, personal communication). DMDS and DMTS probably arise from the chemical oxidation of methanethiol (Parliment et al. 1982; Chin and Lindsay 1994; Yvon and Rijnen 2001). Recently, it was shown, that in the presence of PLP, methanethiol can also be generated chemically (Wolle et al. 2006). In this study, CGL showed demethiolation activity on methionine and the amount of methanethiol produced was considerably higher than its formation in the absence of the enzyme. Based on these results, we propose that *cgl* of *O. oeni* is involved in the transsulphuration pathways of cystathionine, cysteine, homocysteine and methionine (Fig. 5).

Although methanethiol was formed from methionine, the affinity of the enzyme towards methionine was much lower

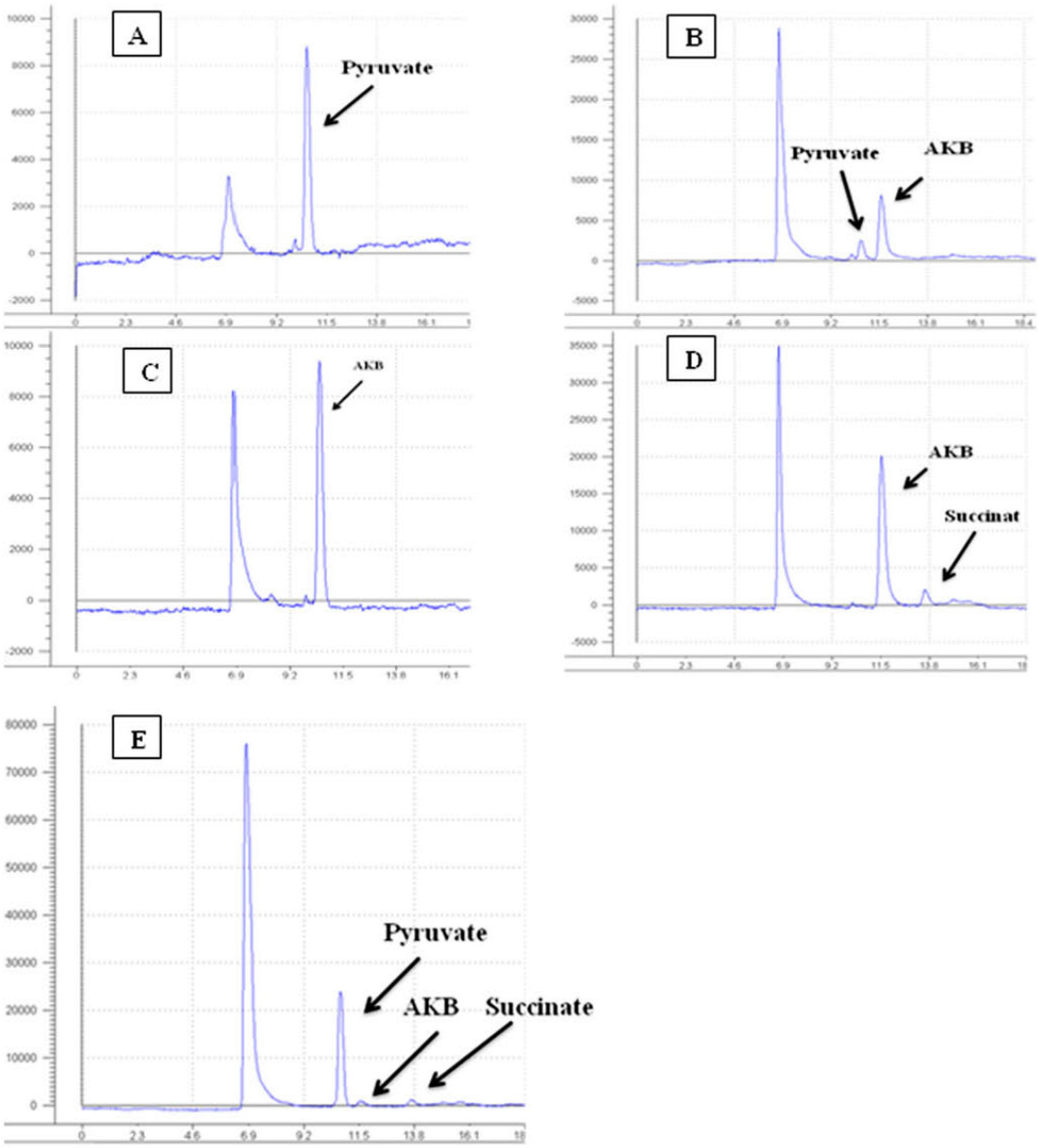

Fig. 3 HPLC analysis of assays carried out with purified enzyme (CGL05) at pH 6.8 for 30 min and L-cysteine (**a**), L-cystathionine (**b**), DL-homocysteine (**c**), *O*-succinyl-*L*-homoserine (**d**), and *O*-succinyl-*L*-homoserine and L-cysteine (**e**). Similar results were obtained with CGL06 (data not shown). Peaks were identified by retention time. *AKB*, α-ketobutyrate

than towards cystathionine. This has been observed before in *Lactococcus lactis* spp *cremoris* (Bruinenberg et al. 1997) and *Lactobacillus casei* (Irmler et al. 2009). Previous studies showed that for both strains used in this study, methionine is essential for growth (Krieger et al., unpublished). This implies, they either lack the enzymes needed for biosynthesis of this amino acid or the pathways are interrupted. Moreover, it was shown that an addition of

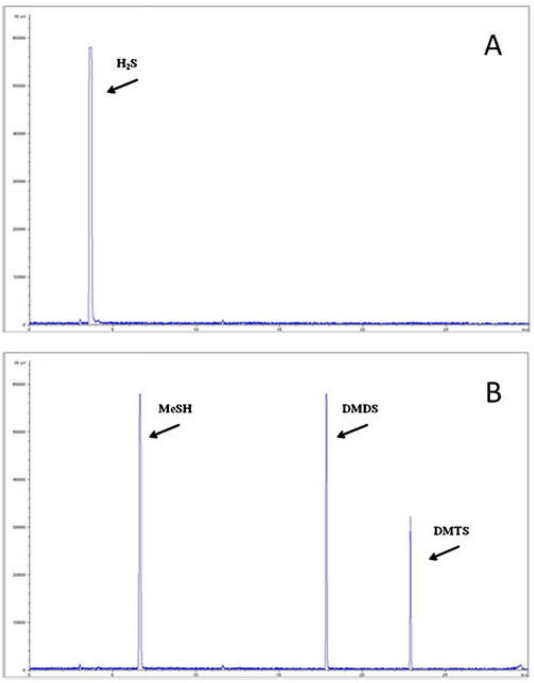

Fig. 4 Head space analysis by GC-PFPD of enzymatic assays carried out with cystathionine at pH 6.8 with the recombinant CGL05. Similar results were obtained with CGL06 (data not shown). *H_2S* hydrogen sulfide (**a**) with methionine at pH 5.5 (**b**). *MeSH*, methanethiol

cysteine and glutathione to a wine after alcoholic fermentation can promote the growth of LAB and MLF (Rauhut et al. 2004); however, this effect seemed to be influenced by the substrate concentration and by the general nutrient composition of the wine (Rauhut et al. 2004). VSC production by whole cells of oenococci has not been broadly studied in literature. It was shown that *O. oeni* is able to produce VSC such as methanethiol, dimethyldisulphide, methional, methionol and 3-(methylthio)-propionic acid (Pripis-Nicolau et al. 2004; Rauhut et al. 2008a, b); however, the increased production of these compounds was observed at substrate concentrations far over the usual levels found in wine after alcoholic fermentation. Furthermore, Rauhut et al. (2008a, b) observed that the catabolism of methionine to VSC was affected by the pH value of the media. Since free sulphur-containing amino acids are usually deficient in wine after alcoholic fermentation, production of these enzymes in oenococci would be suppressed in the wine environment. Therefore, their contribution to VSC is probably minimal. According to Pripis-Nicolau et al. (2004), no increase of VSC which are associated with off-flavours could be detected in wine or wine-like media after the addition of methionine in amounts that can be generally expected in wines. Yeast on the other hand, can synthesise these sulphur-containing amino acids through sulphate and sulphite reduction sequence pathway from inorganic sources such as sulphate and sulphite, which are usually abundant in grape must (Moreira et al. 2002; Swiegers and Pretorius 2007). Moreover, VSC can be produced from other sulphur sources like bisulphite added to the must or from precursors present in grapes. It was demonstrated that some vinification practices could result in an increased formation of VSC (Karagiannis and Lanaridis 1999; Ribéreau-Gayon et al. 2000; Zoecklein 2007).

A better knowledge of the mechanisms of the production of VSC and the genes encoding the enzymes involved is important to improve the understanding of how LAB contribute to the wine aroma during MLF and is therefore a major interest to maintain the quality of wines. Climate change and vinification practices have frequently resulted in wines with high alcohol concentrations [>13% (*v*/*v*)]. In our study, ethanol contents up to 15% (*v*/*v*) had no impact on the enzyme activity. Moreover, the enzymes were stable at temperatures suitable for the wine production and storage. Based on the findings reported in this study, it remains questionable whether the CGL will retain at least partial activity under the harsh wine making conditions since the *cgl* products became inactive at wine-like pH levels. Further work with natural substrates and whole cells of *O. oeni* will be necessary to determine its influence on the VSC production in wine.

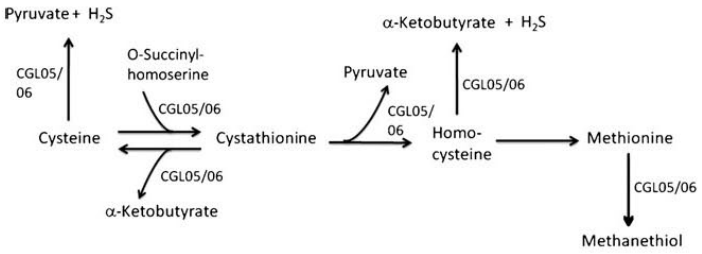

Fig. 5 Proposed catabolic pathways of cystathionine, cysteine and methionine

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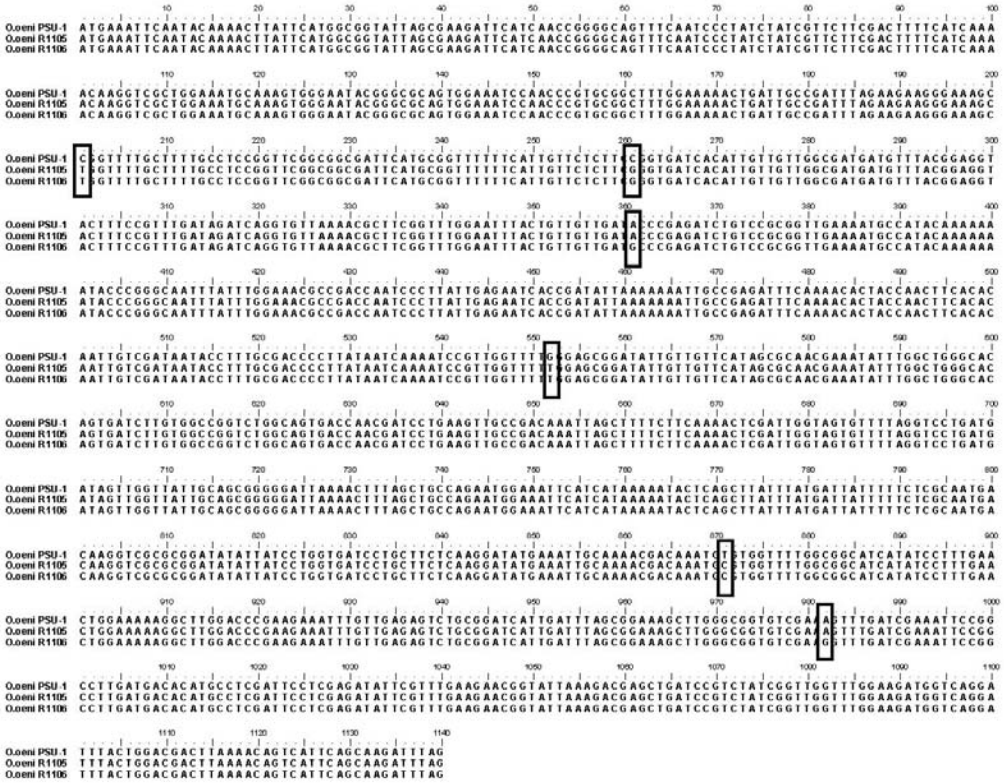

Online source 1 Nucleotide sequence alignment of *cgl* from *O. oeni* PSU-1, R1105 and R1106. The differences are highlighted in boxes.

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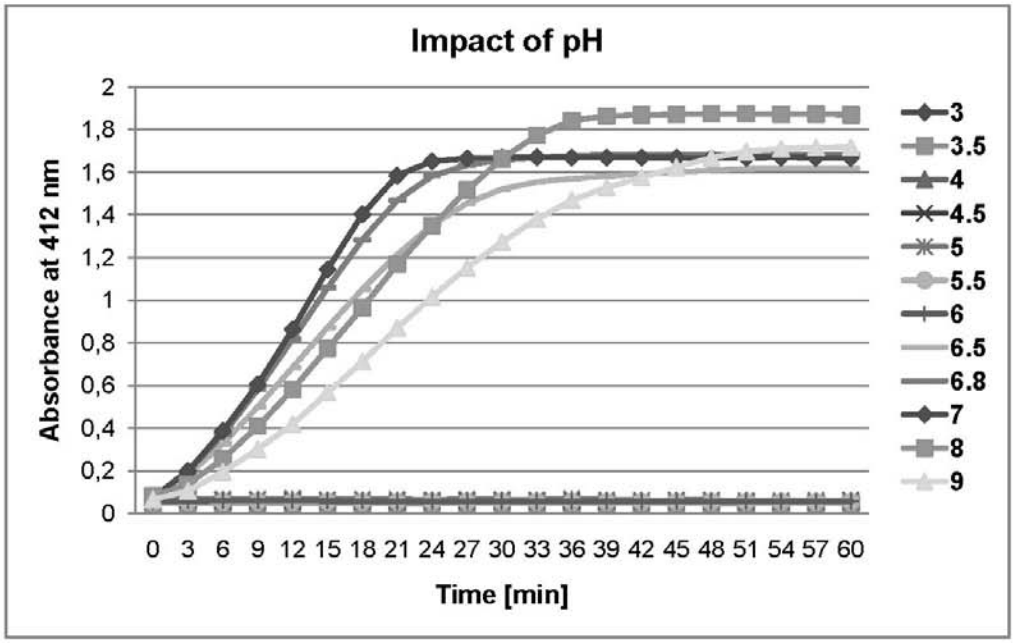
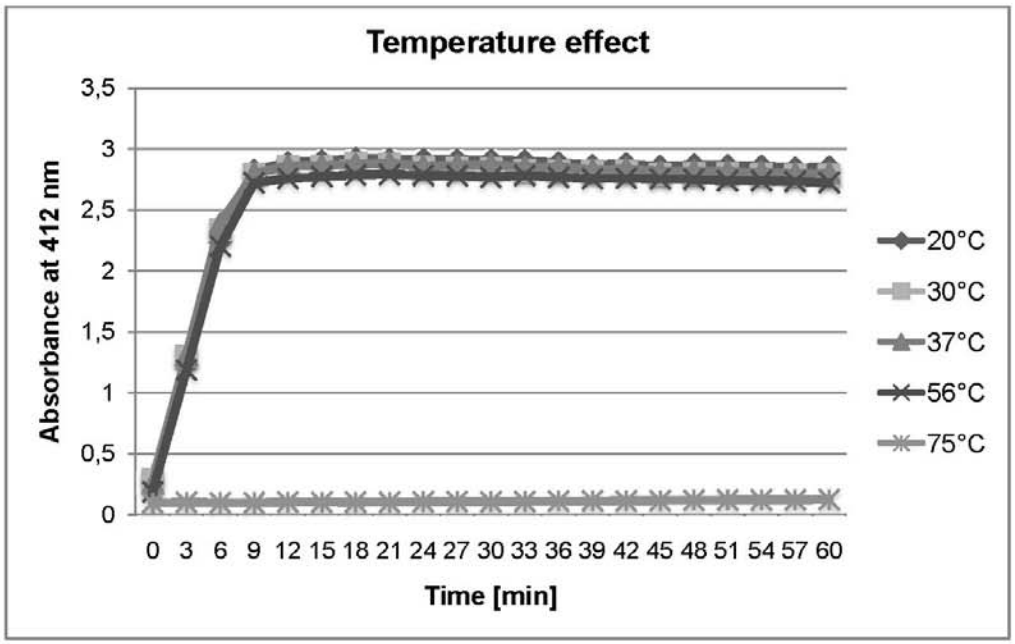


Online source 2 The impact of pH on the activity of the recombinant CGL05. Similar results were obtained with CGL06 (data not shown). The recombinant enzyme was incubated for 30 min at 37°C with 2 mM L-cystathionine.



Online source 3 The impact of temperature on the activity of the recombinant CGL06. Similar results were obtained with CGL05 (data not shown). The recombinant enzyme was incubated for 30 min at five temperatures across the range of 20°C to 75°C.

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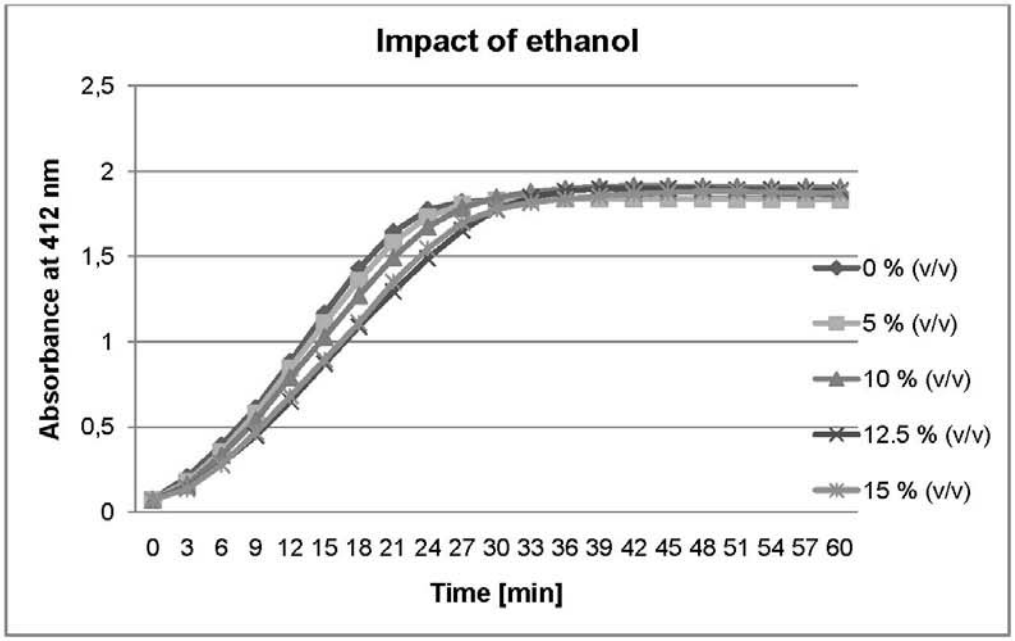

Online source 4 The impact of ethanol on the activity of the recombinant CGL05. Similar results were obtained with CGL06 (data not shown). The recombinant enzyme was incubated for 30 min at 37°C with 2 mM L-cystathionine.

3 Discussion

Various review articles have documented the oenological importance of MLF (Davis et al. 1985; Henick-Kling 1995; Versari et al. 1999; Liu 2002; Lonvaud-Funel et al. 2002; Bartowsky and Henschke 2004; Lerm et al. 2010). During this process L-malic acid is converted to L-lactic acid and CO_2. There are three main reasons for conducting MLF in wine. Firstly, the deacidification of the wine, secondly, contribution to the microbial stability by the removal of malic acid as a possible carbon source and thirdly, the alteration of the wine aroma profile (Volschenk et al. 2006).

It can be conducted by a number of LAB occurring naturally in the must and wine flora. However, commercial available bacterial starter cultures (mostly *O. oeni*) are more and more used to improve and assure the success of MLF. These strains vary in their malolactic activity and growth characteristics. Sought-after attributes among strains include viability at low pH, resistance to high ethanol content and SO_2, no production of biogenic amines or off-flavours and compatibility with yeast strains.

There are a number of reports showing changes in the volatile aroma profile of wines after MLF, summarised by Lerm et al. (2010) and especially in warm climate regions, flavour changes from MLF are of greater importance to the winemaker than the acid reduction (Henick-Kling and Acree 1998). However, these studies were mostly conducted in a synthetic model wine solution, in red wines (e.g. Merlot, Cabernet Sauvignon or Tannat) or in Chardonnay, being the main white cultivar used. Reports which investigated the impact of MLF in Riesling wines from cool climate regions are limited.

The overall objective of this work was to address the impact of partial and complete MLF on the volatile aroma composition of white wines that were produced under small scale standardised experimental conditions. This is also the first study cloning and characterising a cystathionine β/γ-lyase from two *O. oeni* oenological strains.

The first aim was to investigate the influence of the stress factors pH and ethanol on two *O. oeni* strains and the volatile aroma composition of the white wines Riesling and Chardonnay. Currently, climate change and vinification practices frequently result in

wines with higher ethanol concentrations (> 13 % (v/v)) (Mira de Orduña 2010). A combination of inhibiting factors, such as pH and ethanol, may not only result in difficult MLF in hot climate regions in the future, but also in cool climate regions where moderately raised ethanol levels may lead to inhibition in conjunction with low pH values (Mira de Orduña 2010).

The present study showed that the pH and ethanol content as well as the cultivar have a great impact on the success of MLF (Tables 2 and 3 in Paper 1) and the volatile aroma composition of the wines following MLF (Tables 4 and 5 in Paper 1). Moreover, the bacterial strains (*O. oeni* R1105 and R1106) used, differed with regard to their pH and alcohol optimum, thus strain R1105 exhibited better resistance to high ethanol content and low pH values. Ethanol content and low pH are crucial factors limiting bacterial growth (Henick-Kling 1993) and results of this work proved that MLF was concluded faster in the treatments with lower selective pressure.

A pH value of 3.2 combined with 15 % (v/v) ethanol had the greatest inhibitory effect on the bacterial strains tested. This correlates with previous studies (Gockowiak and Henschke 2003; Solieri et al. 2010). Solieri et al. (2010) showed that low pH values are the most negative attribute influencing the malolactic activity. Gockowiak and Henschke (2003) observed that pH in the range 2.9 – 3.5 had a generally large influence on bacterial viability and MLF, whereas alcohol concentration in the range of 12.5 to 14.5 % (v/v) had a lesser impact. While a pH of 3.5 allowed MLF completeness by three of the four strains independently from ethanol content, pH values of 3.0 and 3.2, combined with 10 % and 13 % (v/v) ethanol respectively, were inhibitory for all tested strains (Solieri et al. 2010).

Esterase activity of wine-associated bacterial strains is not well understood (Liu 2002) and it appears that their growth in grape juice and wine might modify the ester profile of wine (Matthews et al. 2004; Matthews et al. 2007; Sumby et al. 2009). Increases of ester concentrations in wine following MLF, including acetic acid ethylester, lactic acid ethylester, succinic acid ethylester (Maicas et al. 1999; Ugliano and Moio 2005), as well as decreases in some esters have been observed previously (Laurent et al. 1994; Bartowsky et al. 2008). Comparative studies concerning the influence of different LAB on the concentration of wine volatile aroma compounds often focus on selected groups of compounds whereas the cultivars tested are often red grapes. The present study focused

on white cultivars and levels of short-chain esters, such as lactic acid ethylester, acetic acid ethylester and succinic acid diethylester, presented higher concentrations after complete MLF but also in the wines with partial MLF. Lactic acid ethylester and acetic acid ethylester were quantitatively the predominant esters in all treatments (Tables 4 and 5 in Paper 1).

Bartowsky et al. (2010) observed changes in the volatile fermentation-derived compounds at different wine pH (pH 3.3, 3.7) in red wines. A lower pH resulted in greater increases in total fruity esters which were reflected in sensorial analysis. In the present work, these observations were confirmed for the white wines, Riesling and Chardonnay (Figure 1 in Paper 1). At low pH (pH 3.2) greater increases of concentrations of e.g. lactic acid ethylester, propionic acid ethylester or succinic acid diethylester were detected, while at the higher pH levels (pH 3.6, 3.8) increased contents of acetic acid 3-methylbutylester or acetic acid phenylethylester were observed. Succinic acid diethylester, associated with fruity and floral notes, contributes significantly to the wine aroma (Clarke and Bakker 2004) and previous studies reported an increase during aging (Jackson 2000). However, other authors have observed increased concentrations after MLF at wine pH 3.2 to 3.7, depending on the bacterial strain used (Davis et al. 1985; Herjavec et al. 2001; du Plessis et al. 2002; Ugliano and Moio 2005; Boido et al. 2009; Lee et al. 2009).

In our study, the highest content of succinic acid diethylester was found in the wines with pH 3.2 and also in the wines with partial MLF where the bacterial population was still present at CFU/mL of 10^2 to 10^4. Generally, tendencies of decreasing ethyl – and acetate ester concentrations could be noted with increasing pH. The ethanol level of the wine on the other hand, seemed to have an impact on the concentration of hexanol or decanoic – and hexanoic acid ethylester for example.

The production of other esters, which contribute to pleasant fruity notes, showed significant differences after MLF depending on wine condition and strain. The total amounts of esters found after MLF in the Riesling and Chardonnay wines suggest their beneficial contribution to the wine's final aroma. Overall the Chardonnay wines contained higher total ester and higher alcohol concentrations than the Riesling wines.

Poor adaptation of the *O. oeni* population in the particularly difficult conditions of fermentation due to the composition of these white wines led to a premature halt in MLF. However, bacterial populations between 10^2 and 10^4 CFU/mL were still present in these wines (Tables 2 and 3 in Paper 1).

Results demonstrated that not only chemical reactions, but also enzymatic actions were present and contributed to the wine aroma, even if MLF was only partially conducted. Thus, it was noted, that for example Chardonnay and Riesling treatments, which have undergone only partial MLF, showed nevertheless increases in fruity esters such as acetic acid ethylester or butyric acid ethylester. In the Riesling treatments with partial MLF, the terpenes trans-linalool oxide, cis-linalool oxide and α-terpineol were also present at higher concentrations, especially at low pH (Table 4 in Paper 1). Ugliano et al. (2003) also observed a significant increase in the concentration of total free terpenols at low pH. The latter authors noted that the hydrolysis of aroma precursors appeared to be strongly enhanced by the occurrence of MLF by comparing the fermented with the unfermented samples which contained low concentrations of terpenols, probably as a result of slow acid catalysed hydrolysis. Also, Sefton (1998) reported that chemical acid hydrolysis is fairly slow under typical vinification conditions. The results of Hernandez-Orte et al. (2009) indicated that *O. oeni* and *Lactobacillus* strains were able to induce clear changes on the volatile profile derived from grape flavour precursors in a model wine system, although only the *O. oeni* strains conducted MLF. They suggested that MLF metabolism may not be linked to the abilities of LAB to hydrolyse and release glycosidically bound aroma compounds.

Medium chained fatty acids, such as decanoic acid, can affect bacterial growth (Lonvaud-Funel et al. 1988) and this negative impact is highly dependent on wine pH, with being more inhibitory at lower pH values (Alexandre et al. 2004). In addition to limiting bacterial growth, medium chain fatty acids can reduce the ability to catabolise malic acid (Capucho and San Romão 1994), which in turn leads to an increase of the duration of MLF (Lerm et al. 2010). The fatty acids act as protonophor and thereby diminish the transmembrane proton gradient which is essential for ATPase activity and transport of metabolites across the cell membrane (Capucho and San Romão 1994; Carreté et al. 2002). According to a hypothesis of Bartowsky (oral presentation, WAC2011), longer chained acids may have an inhibitive impact on LAB, especially at low pH, therefore an

elevated ester metabolism may reflect a stress response, in order to gain a physiological advantage.

The pH strongly affects the survival and the malolactic activity of *O. oeni*, especially in white wines with low pH values, successful MLF is often difficult to achieve. The next step in this work was to evaluate the impact of different inoculation strategies in Riesling wine with high acidity on MLF and the volatile aroma composition.

There are still contradictory opinions regarding the optimal inoculation time for MLF. Co-inoculation is often associated with antagonistic interactions between yeast and bacteria (Alexandre et al. 2004) or off-flavour production, such as excessive amounts of acetic acid (Henick-Kling 1993; Lonvaud-Funel 1999). On the other hand with consecutive MLF, nutrient depletion and inhibiting ethanol concentrations display a harsh environment for the bacterial flora.

To our knowledge, the present study represents for the first time the impact of different bacterial inoculation timings on the MLF performance and the production of volatile aroma compounds in low pH Riesling must. The results proved that it is possible to inoculate the bacterial culture at different timings of AF without, on the one hand, inhibiting AF or, on the other hand, causing the failure of MLF.

However, pH values and the timing of bacterial inoculation were shown to be important for how rapidly MLF commences. These results are in agreement with a previous study (Rosi et al. 2003), carried out in a commercial white grape juice, reporting the possible inoculation with LAB at the beginning, middle, and end of AF without slowing down or inhibiting AF or causing failure of MLF. Yet, at pH 3.2 a lowering of bacterial viability was observed (Rosi et al. 2003).

In comparison to the consecutive treatments, bacterial inoculation 24 h after yeast addition or at 60 % of AF, proved to be a better option to overcome inhibiting low wine pH and high ethanol content in the present study.

The co-inoculation at 40 % of AF seemed to be a less optimal inoculation point during AF, as the pH of the fermenting must was further reduced as a result of acid production by yeast metabolism (Table 1 in Paper 2). Similar observations were made by Rosi et al. (2003). These authors suggested that at the halfway stage of AF, the antago-

nistic effect of yeast toward bacteria may be strongest due to SO_2 accumulation, ethanol or acid production as well as other toxic metabolites and it cannot be guaranteed that the bacteria can defeat this antagonism.

In the present study MLF was concluded in all treatments and a general reduction in total fermentation time could be achieved using simultaneous inoculation techniques. The time gained ranged between 25 and 50 days, depending on the bacterial strain and inoculation time used. A reduction on total fermentation time allows earlier and immediate downstream treatments such as racking, fining, and SO_2 addition, thus increasing microbial stability and processing efficiency. However, when *O. oeni* R1124 was used, the length of MLF itself in the treatments with simultaneous inoculation was similar or longer than their respective sequential treatment. *O. oeni* R1105, on the other hand, carried out MLF faster in the simultaneous treatments than in the consecutive ones and was generally less inhibited by the low pH than R1124. *O. oeni* R1124 seems to be better suited for a sequential MLF, while strain R1105 can be used for both, co-inoculation and sequential MLF. The same strain tendencies were observed in other studies conducted in red wine (personal communication Krieger-Weber, 2011).

Wine chemical composition plays an important role in the metabolism of *O. oeni* during MLF. At lower wine pH (pH <3.5), it will metabolise organic acids in preference to sugars to gain energy (ATP) (Ribéreau-Gayon et al. 2006). Conversely, at higher wine pH (pH >3.7), *O. oeni* will preferentially metabolise sugars which may lead to a higher increase of volatile acidity (Bartowsky et al. 2010). The benefits and risks of sequential and simultaneous AF / MLF remain controversial. No negative effect on final wine quality could be substantiated in this study.

It was suggested that simultaneous inoculation of yeast and bacteria could result in increased concentration of acetic acid produced by LAB in the presence of available sugars in the must (Davis et al. 1985). In the co-inoculation treatments, levels of acetic acid never exceeded 0.7 g/L, considering that the wines without bacterial inoculation contained levels between 0.5 and 0.8 g/L. This confirmed the results of other studies (Semon et al. 2001; Jussier et al. 2006; Massera et al. 2009), demonstrating the possibility of simultaneous fermentation without excessive increase of volatile acidity.

Most investigations on simultaneous inoculation, evaluated yeast-bacteria-interactions and focused mainly on acetic acid and diacetyl production (Semon et al. 2001; Rosi et al. 2003; Jussier et al. 2006). However, little is known about the impact of simultaneous inoculation on the production of volatile aroma compounds. Based on sensorial data it was suggested that Chardonnay (Jussier et al. 2006), Malbec (Massera et al. 2009) and Shiraz wines (Bartowsky et al. 2002) fermented with co-inoculation tend to be fruitier than the wines with sequential inoculation.

In the present work, the Riesling wines with sequential inoculation contained the lowest concentration of acetate and ethyl esters, most notably due to lower concentrations of acetic acid phenylethylester, acetic acid 3-methylbutylester, butyric acid ethylester, lactic acid ethylester and succinic acid diethylester (Table 3 in Paper 2). This might potentially result in a reduction of the fruitiness of the wines. The treatments with the 24 h co-inoculation exhibited the highest content of fruity ethyl esters. Additionally, changes in the ester profile were affected by the bacterial strain used. For example, *O. oeni* R1105 tended to produce more lactic acid ethylester, which is associated with fruitiness, milky notes and an increased mouthfeel. Strain dependency was also observed in the previous experiment and by other authors (Delaquis et al. 2000; Pozo-Bayón et al. 2005; Ugliano and Moio 2005; Boido et al. 2009).

Comparison of fermentation-derived compounds from treatments with simultaneous and sequentially inoculated MLF, has illustrated that the profiles of the wines produced, were very different as a result of the MLF inoculation regime and *O. oeni* strain. The profiles of fermentation-derived compounds of the wines that conducted MLF are clearly distinguishable from those that did not. In addition, wines with complete MLF could be clearly separated according to the inoculation timing and could be distinguished from the wine without MLF. Also, the treatments inoculated for MLF at 24 h and 40 % of AF could be further separated according to the bacterial strain used.

Another important group of volatile aroma compounds in wine are VSC. These potent flavour components occur at very low concentration, have very low sensory threshold values, and are usually associated with negative odours such as ‘cabbage’, ‘rotten egg’, ‘onion’ or ‘rubber’ (Rauhut 1993; Mestres et al. 2000). The only extensive research on

sulphur metabolism has been carried out on the enzymes produced in dairy-associated LAB (Weimer et al. 1999; Seefeldt and Weimer 2000; Yvon and Rijnen 2001; van Kranenburg et al. 2002).

Pripis-Nicolau et al. (2004) investigated for the first time the methionine catabolism of *O. oeni* under vinification conditions. The latter authors noted an increased concentration of 3-(methylsulphanyl) propionic acid, associated with chocolate and roasted odours, in red wines following MLF. Moreover, no off-flavour compounds like methanethiol and dimethyl disulphide could be detected in the wines following MLF. This is in accordance with the present study.

Nevertheless, knowledge of the mechanisms involved in the production of VSC in *O. oeni* and the genes encoding the participating enzymes, is critical to enhance the understanding of how bacteria impart their impact during vinification.

The available genomic information provides additional possibilities to study the flavour-forming potential of LAB. But, biochemical methods are necessary to investigate enzymatic function, substrate specificity and the activity of the genes of interest.

Since methionine and cysteine are generally present in only limited quantities in wine, the formation of VSCs will depend on both the biosynthesis and catabolic pathways of methionine and cysteine. However, the metabolism of sulphur-containing amino acids is diverse, especially considering the existence of multiple alternative pathways as well as several possible chemical reactions which also can contribute to VSC formation.

This work identified an enzyme that degrades sulphur-containing amino acids from two *O. oeni* strains of oenological origins. The genes encoding a cystathionine lyase were highly conserved among three compared *O. oeni* strains (Online Source 1 in Paper 3).

Applying comparative sequence analysis, Liu et al. (2008) revealed that genes encoding cystathionine lyases fall into two distinct families named CBL/CGL and CBL, which share little sequence similarity. The *in silico* translation of *cgl* of *O. oeni* was compared with other known and predicted CBL/CGL sequences using NCBI protein blast.

Besides being nearly identical with the CGL (OEOE_1758) of *O. oeni* PSU-1, it showed 73 % identity with the CBL/CGL of *L. salvarius* UCC118 and ATCC 11741; 72 % with the CGL of *L. reuteri* DSM 20016, and 70 % with *L. fermentum* ATCC 14931. Furthermore, it showed 67% identity with Ctl1 of *L. casei* FAM18168 67% with Ctl2 of *L.*

casei FAM18108, 60% with MetC of *L. lactis* subsp. *cremoris* SK11, and 55% with YrhB of *Bacillus subtilis* str. 168.

The latter four genes also belong to the CBL/CGL family and have experimentally been shown to encode a cystathionine lyase exhibiting dual CBL/CGL activity.

Probably all enzymes belonging to "CBL/CGL" may display a mixture of cystathionine β- and γ-lyase activities (Liu et al. 2008). It implies that LAB enzymes in this subcluster could have either solo CGL activity or a dual CBL/CGL activity (Liu et al. 2005).

In this study, the gene product of *cgl* also showed dual CBL/CGL. It has features of a cystathionine-γ-lyase (EC 4.4.1.1), a pyridoxal-5-phosphate-dependent enzyme catalyzing an α,γ-elimination reaction of L-cystathionine to produce L-cysteine, α-ketobutyrate and ammonia. Moreover, it was able to catalyse an α,β-elimination reaction producing homocysteine, pyruvate and ammonia from L-cystathionine. An elimination reaction of L-cysteine and DL-homocysteine was also efficiently catalysed by the enzyme, resulting in the formation of hydrogen sulphide. However, the enzymes exhibit cystathionine γ-synthase activity when *O*-succinyl-L-homoserine and L-cysteine are present. This attribute has also been reported for *MetB* of *Lactobacillus casei* (Irmler et al. 2008) and was found for Ctl1 (S. Irmler, personal communication). Furthermore, the ability to demethiolate methionine into methanethiol, an unfavourable volatile sulphur compound in terms of wine aroma, was observed. The amount produced, was considerably higher than its formation without the enzyme.

Based on these findings, we propose that *cgl* of *O. oeni* is involved in the transsulphuration pathways of cystathionine, cysteine, homocysteine and methionine (Figure 5 in Paper 3). In our study, ethanol contents up to 15 % (v/v) had no impact on the activity of the purified enzymes (Online Source 4 in Paper 3). Moreover, the enzymes were stable at temperatures suitable for the wine production and storage (Online Source 3 in Paper 3). By using L-cystathionine as substrate, the enzyme activity was highest at pH 8.0 (Online Source 2 in Paper 3). No activity was observed at a pH below 6.5. In contrast, L-methionine was degraded at pH 5.5 and 6.

It was shown that an addition of cysteine and glutathione to a wine after alcoholic fermentation can promote the growth of LAB and MLF (Rauhut et al. 2004); however, this effect seemed to be influenced by the substrate concentration and by the general nutrient

composition of the wine (Rauhut et al. 2004). Furthermore, Rauhut et al. (2008a; 2008b) observed that the catabolism of methionine to VSC was affected by the pH of the media.

Yet, an increased production of VSC by *O. oeni* was only observed at substrate concentrations far over the usual content found in wine after alcoholic fermentation (Pripis-Nicolau et al. 2004; Rauhut et al. 2008a; Rauhut et al. 2008b). Since free sulphur-containing amino acids are usually deficient in wine, production of these enzymes in oenococci would be suppressed in the wine environment, thus their contribution to VSC is probably minimal. It was proposed that other factors such as the chemical or biochemical transformation of other volatile or non-volatile sulphur precursors in wine may be the reason for 'reductive' sulphur off-flavours which occur occasionally in wines following MLF (Rauhut 2009).

Due to its complex nature, wine is subject to permanent changes in composition and therefore there is an invaluable role for continuing investigations into causes and interactions that result in increases of desirable and undesirable wine flavours.

While grape juice is the origin, it is the metabolism of grape compounds by yeast and bacteria that is essential for the development of wine flavour (Bartowsky and Pretorius 2009).

This work has sought to provide new knowledge of bacterial and chemical interactions that contribute to the flavour and quality of Riesling and Chardonnay wines. This research provides information which is both of fundamental and industrial importance and proves that MLF is more than deacidification.

It is evident that modifications in flavour profiles during MLF are not only dependent on the bacterial strain conducting MLF, but also on the grape cultivar, the chemical composition of the wine (especially pH and ethanol content), as well as the timing of inoculation. LAB strains used in the study responded differently to the wine conditions, suggesting that LAB vary in tolerance to various stresses in the wine environment. This phenotypic variability is linked to genotypic differences (Bridier et al. 2010). They may be characterised by the presence or absence of some genes (Renouf et al. 2008) which

could be implicated in the response to environmental stress or by the variation in gene expression (Beltramo et al. 2006; Olguín et al. 2010).

Although, the strains used in this work, revealed differences in their fermentation rate, in the production of volatile aroma compounds and e.g. in the citric acid consumption, they exhibit consistent characteristics and can be used to enhance the fruitiness of white wines. It was also observed, that not each *O. oeni* strain can be used as 'all in one solution', but has to be carefully selected according to wine chemical composition and inoculation scenario.

Results indicate that even partial MLF has distinct influences on the aroma profile. In addition, it was observed that the impact of simultaneous inoculation on the fermentation success and on the final wine was not negative to the quality. Applying a co-inoculation protocol may offer microbiological, technological and sensorial advantages, especially in low-pH, cool-climate white musts with potential high ethanol content.

Research has shown that LAB possess a broad range of ester synthesising and hydrolysing abilities, many of which may affect wine composition and organoleptic properties. A better understanding of these bacterial activities is of great interest, as new techniques for altering wine aroma could be developed.

Future research will also benefit from a complete sensorial evaluation including descriptive analysis, which will further enhance the knowledge available on the aroma modifications associated with MLF.

4 Summary

Malolactic fermentation (MLF) is a biochemical process typically occurring in the vinification process after completion of alcoholic fermentation (AF), consisting of the conversion of L-malic acid into L-lactic acid and CO_2. It can be conducted by different species of lactic acid bacteria (LAB), among which *Oenococcus oeni* is the most often associated with MLF in the harsh wine environment due to its tolerance and adaptation to high acidity and alcohol contents in wine. Various stress factors in wine such as low pH and high ethanol concentrations affect the growth of LAB or their metabolic properties and consequently the timely completion of MLF.

The overall objective of this work was to address the impact of partial and complete MLF on the volatile aroma composition of white wines. The first aim was to investigate the influence of the stress factors pH and ethanol on two *O. oeni* strains and the volatile aroma composition of white wines from the grape varieties Riesling and Chardonnay.

It was demonstrated that the wine matrix as well as the pH and alcohol concentration affect MLF and the final volatile aroma profile. Results indicate that changes in the volatile aroma composition are not necessarily related to complete MLF and that even partial MLF has distinct influences on the wine aroma profile of white wines.

The next step in this study was to evaluate the impact of four different inoculation strategies in Riesling wine with high acidity on MLF and the volatile aroma composition. Treatments with simultaneous inoculation showed a reduced total fermentation time (alcoholic and malolactic) compared to the sequential inoculations. It was observed that simultaneous alcoholic and malolactic fermentation had no negative impact on fermentation success and on the final wine volatile aroma composition. Compared to sequential inoculation, wines with co-inoculation tended to have higher concentrations of ethyl and acetate ester which may result in fruitier wines.

This is also the first study cloning and characterising a cystathionine β/γ-lyase from two *O. oeni* oenological strains. Biochemical characterisation led to the conclusion that the enzyme is a multifunctional pyridoxal-5'-phosphate (PLP)-dependent enzyme that on the one hand degrades cystathionine by an α,β- and an α,γ-elimination reaction and on the other hand exhibits cystathionine γ-synthase activity when *O*-succinyl-L-homoserine

and L-cysteine are present. Furthermore, the ability to demethiolate methionine into methanethiol, an unfavourable volatile sulphur compound in terms of wine aroma, was observed.

The present study has highlighted the role of *O. oeni* in the modification of wine volatile aroma compounds and the impact of pH, ethanol and wine matrix on its metabolic activity.

It was shown that changes of the wine aroma profile during MLF can be affected by the bacterial strain and the MLF inoculation regime as well as the grape cultivar or chemical wine composition. A better understanding of these bacterial activities is of great interest, as it could lead to the development of new techniques for altering the aroma of wine.

5 Zusammenfassung

Die malolaktische Fermentation (MLF), auch als biologischer Säureabbau bezeichnet, ist ein biochemischer Prozess, der im Wein in der Regel nach der alkoholischen Gärung stattfindet. Bei der malolaktischen Fermentation wird L-Äpfelsäure unter Energiegewinnung zur schwächeren L-Milchsäure decarboxyliert. Mehrere Gattungen von Milchsäurebakterien können eine MLF durchführen, *Oenococcus oeni* ist jedoch die am besten an das Weinmedium angepasste Bakterienspezies. Verschiedene, im Wein vorkommende Stressfaktoren, wie z. B. niedrige pH-Werte und hohe Alkoholgehalte, beeinflussen das Bakterienwachstum, ihre Stoffwechselprozesse sowie die zeitige Beendigung der MLF.

Das übergreifende Ziel dieser Arbeit war es, die Auswirkung einer partiellen und vollständigen MLF auf das Aromaprofil von Weißweinen zu untersuchen.

Zunächst wurde der Einfluss zweier Stressfaktoren (pH-Wert und Alkoholgehalt) auf zwei *O. oeni* Stämme und die Bildung flüchtiger Aromastoffe in Weißweinen der Rebsorten Riesling und Chardonnay geprüft. Die Ergebnisse dieser Studie zeigten, dass der pH-Wert und der Alkoholgehalt des Weines sowie die Rebsortenmatrix den Verlauf der MLF und die Bildung flüchtiger Aromastoffe des Weines deutlich beeinflussen. Auch wurde deutlich, dass schon eine partielle MLF Auswirkungen auf das Aromaprofil hat.

Anschließend wurden vier unterschiedliche Beimpfungzeitpunkte für die MLF in Riesling-Weinen mit hohen Säurewerten untersucht. Im Vergleich zur sequentiellen Inokulation, konnte mit einer simultanen Beimpfung eine Reduzierung der gesamten Gärdauer erreicht werden. Die simultane MLF hatte keinen negativen Einfluss auf das Aromaprofil der Weine und zeichnete sich durch erhöhte Esterkonzentrationen aus.

Zusätzlich ist dies auch die erste Studie, die über eine Klonierung und Charakterisierung einer Cystathionin-β/γ-Lyase zweier *O. oeni* Stämme berichtet. Bei dem identifizierten Enzym handelt es sich um ein Pyridoxalphosphat (PLP)-abhängiges Enzym, das einerseits die Fähigkeit besitzt, Cystathionin durch eine α,β- und eine α,γ- Eliminations-Reaktion abzubauen. Anderseits weist dies in Gegenwart von *O*-Succinyl-L-Homoserin und L-Cystein eine Cystathionin γ-Synthase -Aktivität auf. Darüber hinaus besitzt es die Eigenschaft, Methionin zu Methanthiol zu demethylieren.

Diese Arbeit zeigt den Einfluss von *O. oeni* auf das Aromaprofil im Wein in der Abhängigkeit von pH-Wert, Alkoholgehalt und Weinmatrix.

Die Ergebnisse weisen darauf hin, dass die Produktion von flüchtigen Aromastoffen, durch Bakterienstamm, Beimpfungszeitpunkt, Rebsorte und chemische Zusammensetzung des Weines beeinflusst wird. Erkenntnisse über Bakterienstoffwechsel-Aktivitäten sind von großer Bedeutung, da sie dazu dienen können, neue Techniken zu entwickeln, die das Weinaroma positiv verändern.

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7 Addendum A

Fingerprinting MLF wines: Comparison of two analytical techniques

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2 Fingerprinting MLF wines: Comparison of two analytical techniques

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Introduction

Malolactic fermentation (MLF) is a secondary fermentation in the vinification process and is characterised by the conversion of L-malic acid to L-lactic acid and CO_2. It is conducted by lactic acid bacteria (LAB) and may occur spontaneously or be induced by the inoculation of commercial bacterial starter cultures. MLF influences three different, but linked, facets of wine quality: acidity, microbial stability and sensorial complexity of wine. The success of MLF is influenced by several oenological parameters, such as pH, temperature, alcohol content and SO_2 concentration **[1]**.

The metabolic activity, as well as the kinetics of MLF, will influence the sensory profile of the wine linked to the vinification techniques, the physical and chemical composition of the wine. The sensory impact of LAB is less well understood. Swiegers et al. **[2]** listed the possible pathways by which LAB are able to produce volatile compounds by e.g. metabolising grape components or modifying yeast derived secondary metabolites. The analysis of these produced volatile compounds in wine by gas chromatography–mass spectrometry (GC–MS) is time consuming. Rapid sensor techniques, such as electronic noses, are increasingly being used as modern analytical tools. It is well recognised that this technique can effectively represent a 'fingerprint' of the sample being analysed and can reduce analytical time **[3]**.

In order to evaluate the influence of pH and ethanol on the performance of different LAB starter cultures as well as on the volatile profile and wine quality, this study was conducted over three vintages in two different climatical winegrowing regions and three white wine varieties (Chardonnay, Riesling and Sauvignon blanc). The potential of an electronic nose based on mass spectrometry was assessed to differentiate between the MLF starters used and to obtain a fingerprint of the volatile compounds produced by LAB.

Furthermore, the relationship between GC-MS analysis of volatile aroma compounds and an electronic nose based on mass spectrometry was investigated using multivariate data analysis.

Materials and methods

Riesling grapes from Geisenheim wine region (Germany) and Sauvignon blanc grapes originating from Paarl wine region (South Africa) were harvested during the 2008 and Chardonnay grapes originating from Paarl wine region (South Africa) were harvested during the 2009 season. Grape processing and alcoholic fermentation were carried out following standard vinification procedures. Before inoculation with bacterial strains the pH of the Riesling and Chardonnay wines was set to 3.2, 3.6 and 3.8. The alcohol was adjusted to 12.5% (v/v) and to 15% (v/v). Three different bacteria strains, belonging to the genera *O. oeni* (R1105, R1106) and *Lb. plantarum* (R1122), were tested during MLF and were inoculated at approximately 5*106 CFU/mL. All experiments were done in triplicate.
Musts were analysed at the time of crushing and wine samples were collected during and after AF and MLF and analysed by Fourier Transform Infrared (FT-IR) spectroscopy (FOSS) and high performance liquid chromatography (HPLC). HPLC analysis was performed according to Schneider et al. **[4]**. Volatile aroma compounds were detected using gas chromatography - mass spectrometry (GC-MS) according to Rapp et al. [6], modified by Fischer and Rauhut (2005, unpublished). Moreover, an MS-based electronic nose (SMartNose®) was tested according to Irmler et al. **[5]**.

Results and discussion

Chemical analysis of Sauvignon blanc wines

Principal component analysis (PCA) of the major volatile aroma compounds of Sauvignon blanc

wines before and after MLF analysed by GC-MS demonstrated that samples can be grouped according to LAB strain **Figure (1)**.

Figure 2 shows preliminary results of a screening of Sauvignon blanc wines after MLF with an electronic nose. 99% of the variance was explained by the first two principal components. The PCA showed that the samples can be grouped according to the added bacterium indicating that the bacteria contribute to the production of volatile compounds in wine.

Chemical analysis of Riesling and Chardonnay wines

The analyses of volatile aroma compounds showed variations in the aroma profile depending on pH, alcohol content, wine matrix and strain. Moreover, clear changes in the aroma composition before and after MLF were evident.

PCA of fingerprints of the Riesling and Chardonnay wines, inoculated with *O. oeni* R1105, obtained with the electronic nose, illustrated that the samples can be grouped according to pH and cultivar for example **(Figure 3).**

This study showed that there is a relationship between both methods used. This technique can be used as preliminary screening before further analysis involving other analytical techniques such as GC–MS, to determine the flavour profile and sensory qualities of wine, are employed. The relative benefits of using an electronic nose may provide a tool for rapid and objective screening of wines before further GC-MS or sensory analysis. ■

Figure 1: PCA of the major volatile aroma compounds of Sauvignon blanc wines before and after MLF. A – Scores, B – Loadings.

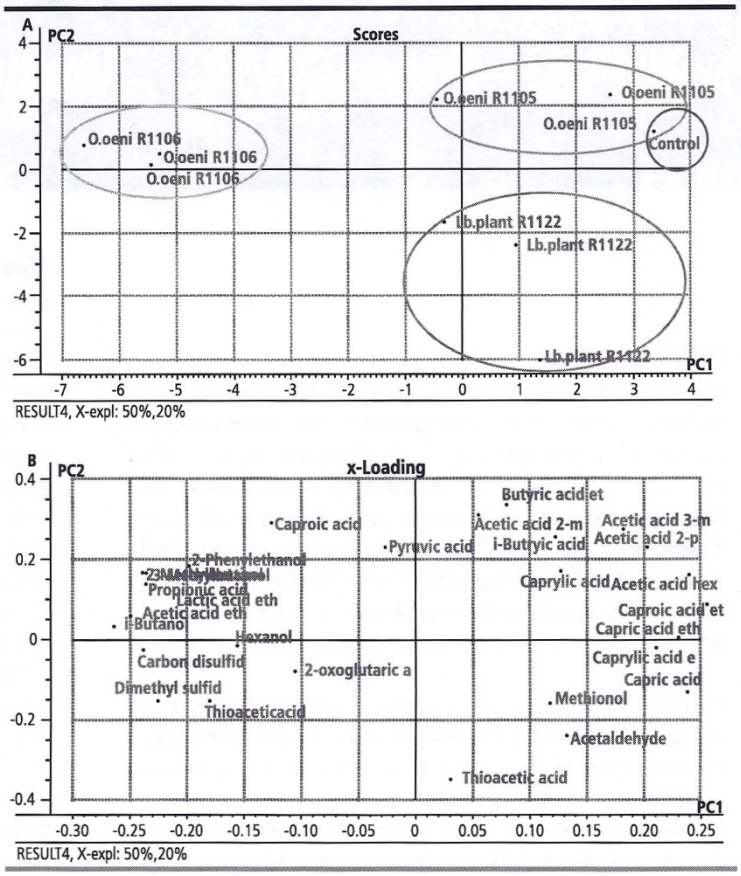


Figure 2: PCA score plots of fingerprints of Sauvignon blanc wines obtained with the SMartNose.

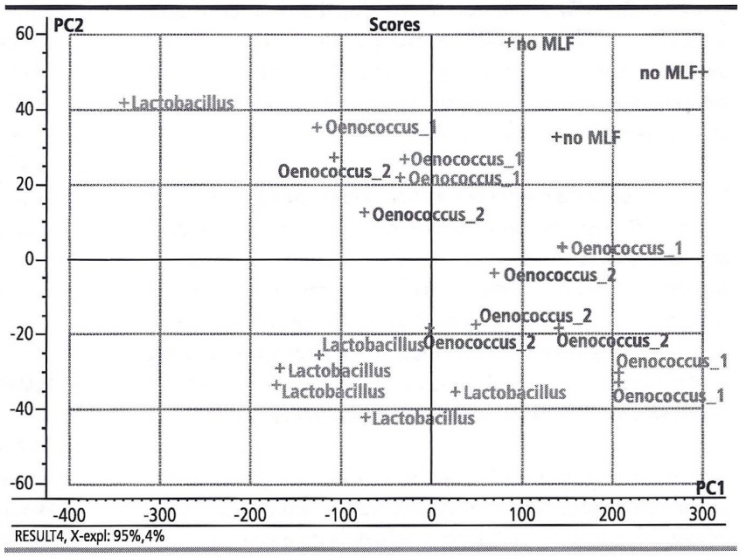
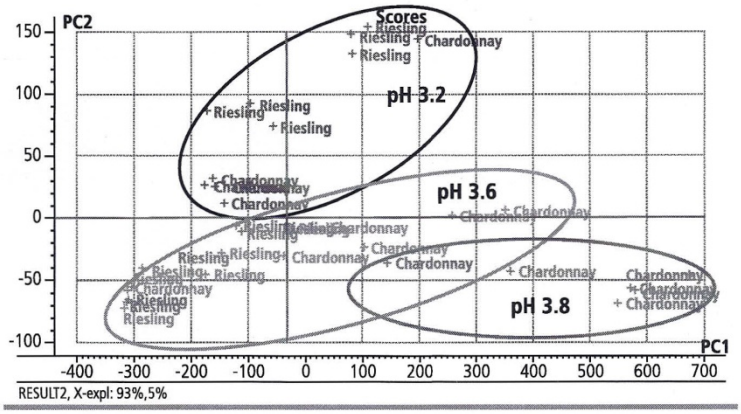


Figure 3: PCA of fingerprints of Riesling and Chardonnay wines with 12.5% (v/v) after MLF with O. oeni R1105.



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8 Addendum B

Sensorial aspects of malolactic fermentation in white wines –

Human nose versus electronic nose

Abstract

Wine is primarily described according to its 'bouquet' and to the odour/aroma element of its flavour on tasting (Clarke and Bakker 2004). Malolactic fermentation (MLF) has been shown to modify the wine aroma profile and the sensory impact of the compounds formed during this process, consequently effect the consumer perception of the wine.

Electronic noses offer an additional technique for analysing aroma and are complementary to sensory analysis. The main difference between the human and electronic nose is that the latter is not able to define what the complex aroma is or whether it is acceptable to the human (Hodgins 1997).

This preliminary study describes the influence of two *O. oeni* strains on the sensory characteristics of experimentally produced white wines (German Riesling and South African Chardonnay) through descriptive analysis. In addition, for comparison, a combination of a mass spectrometry (MS)-based electronic nose (SmartNose®) and chemometrics was explored, to classify the wines according to bacterial strain used. Moreover, the electronic nose was tested to assess, if it can assist in determining the relationship between the chemical composition and sensory characteristics of the wine.

Sensory properties observed in relation to specific bacterial strains used, were found to vary between the Riesling and Chardonnay wines, as well as between the different pH levels. Results indicate that the differences were perceived in terms of aroma and of mouthfeel.

Preliminary results obtained with the electronic nose, showed that a combination of both MS-based electronic nose data and chemometrics methods could not always provide acceptable discrimination between the samples.

This study illustrates the impact of *O. oeni* starter cultures on the wine aroma and highlights the importance of strain selection.

Materials and methods

Wines

The Riesling wines from the harvest season 2008 (Rheingau wine region, Germany) and the Chardonnay wines from 2009 originating from Paarl wine region (South Africa) were used for a first sensorial evaluation and a preliminary analysis with an electronic nose. The preparation of the wines is further described in chapter 2.1.

Only the wines with 12.5 % (v/v) were used for the evaluations. The Riesling wines with pH 3.2 were also excluded from the evaluations, because of the stuck MLF in the wine inoculated with *O. oeni* R1106.

Two different *O. oeni* strains were used for MLF and the sensory attributes of these starter cultures, as described by the specific manufacturers, are listed in Table 1.

Table 1. Sensory attributes of the bacterial cultures according to the manufacturer.

***O. oeni* strain**	**Sensory contribution description in brief**
R1105	Enhances complexity and mouthfeel
R1106	Activity and varietal aroma

Sensory evaluation procedure

Quantitative descriptive analysis was used to measure the intensity of specific sensory attributes. Each wine was evaluated according to five descriptors (Table 2), where each descriptor was rated on an intensity scale from low to high. A card of the descriptors was present at each tasting.

All wines were tasted at room temperature of approximately 20 ºC and were evaluated by sniffing and tasting. The wines were served in clean, dry ISO wine glasses and cov-

ered with Petri dishes in order to retain their aroma. Water and biscuits were given to the judges to refresh their mouth between the wines. Each sample had a two or three digit randomised code which corresponded with the tasting sheet.

Sensory panel

An informal preliminary tasting of the Riesling wines which completed MLF was carried out by a panel consisting of eleven members (post-graduate students and staff members from the Department of Microbiology and Biochemistry, Research Center Geisenheim, Germany).

An informal tasting of the Chardonnay wines which completed MLF was carried out by a panel consisting of nine members (lecturers and post-graduate students from the Department of Viticulture and Oenology and the Institute for Wine Biotechnology, Stellenbosch University, S.A.). The descriptive intensity test was performed in triplicate.

Table 2. Definitions of the five attributes used in the sensory evaluation of the wines.

Attributes	**Definitions**
Buttery/ Yoghurt	Clean, fatty, mild flavour of fresh butter and cream. Butter flavoured popcorn.
Fruity	A mixture of non-specific fruits such as berries (strawberries, raspberries, black currants) or tropical fruits.
Mouthfeel	The overall texture, smoothness or weight of wine in the mouth.
Reduced, reductive	Aromas associated with sulphur compounds that are reminiscent of skunk, rubber, rotten egg, cooked cabbage and onion.
Rancid yoghurt, cheese	At extreme levels yoghurt, sour cream and rancid butter or cheese aromas.

MS-based electronic nose

Moreover, an MS-based electronic nose (SMartNose®) was tested according to Irmler et al. (2006) with following modifications: volatile aroma compounds were extracted by

an INDEx- (Inside Needle Extraction) device filled with Tenax as adsorptive material. Mass spectra were recorded from m/z 10 to 160.

Analysis of data

Multivariate statistical analysis was performed to evaluate the results obtained with the electronic nose, using *The Unscrambler* software (version 9.2, CAMO ASA, Norway). The *PanelCheck* software (version 1.3.2, Nofima, Norway) was used to evaluate panel performance.

Principal component analysis (PCA) was used to investigate the distribution of the wine samples relative to each other based on their sensory attributes.

Results and discussion

An initial preliminary descriptive tasting was done, in order to evaluate whether sensory differences could be perceived between the control wine (wine without MLF) and a MLF wine, fermented with different *O. oeni* strains. Results of the tasting were analysed with the program PanelCheck V1.3.2 to determine whether the panel could discriminate between the different treatments. PCA was performed on the standardised data of the tasting.

Riesling wines

Differentiation in the perceived sensory properties of the two different bacterial strains used in the Riesling wines with pH 3.6 and 3.8 is evident from the PCA biplot and spider plot results shown in Figures 1 and 3. The control sample without MLF was in all cases strongly correlated with fruitiness. *O. oeni* R1105 separated towards buttery, but also rancid yoghurt aroma attributes, while R1106 was correlated with a greater mouthfeel and more reductive flavours.

The SmartNose® on the other hand was not able to clearly distinguish between the wine samples (Figure 2 and 4). A better separation of the bacterial strains used, could be achieved in the wines with pH 3.6 than in the wines with pH 3.8.

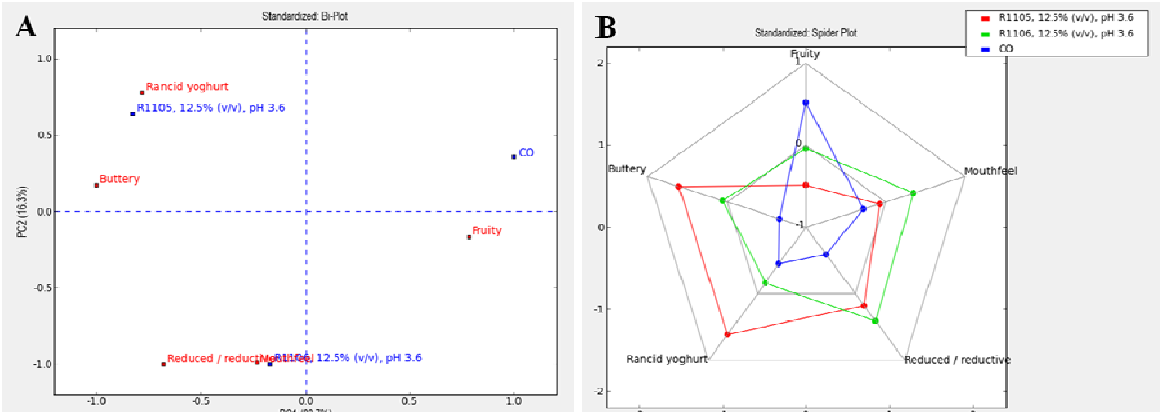

Figure 1. Standardised PCA biplot (A) and standardised spider plot (B) of the Riesling wines with pH 3.6.

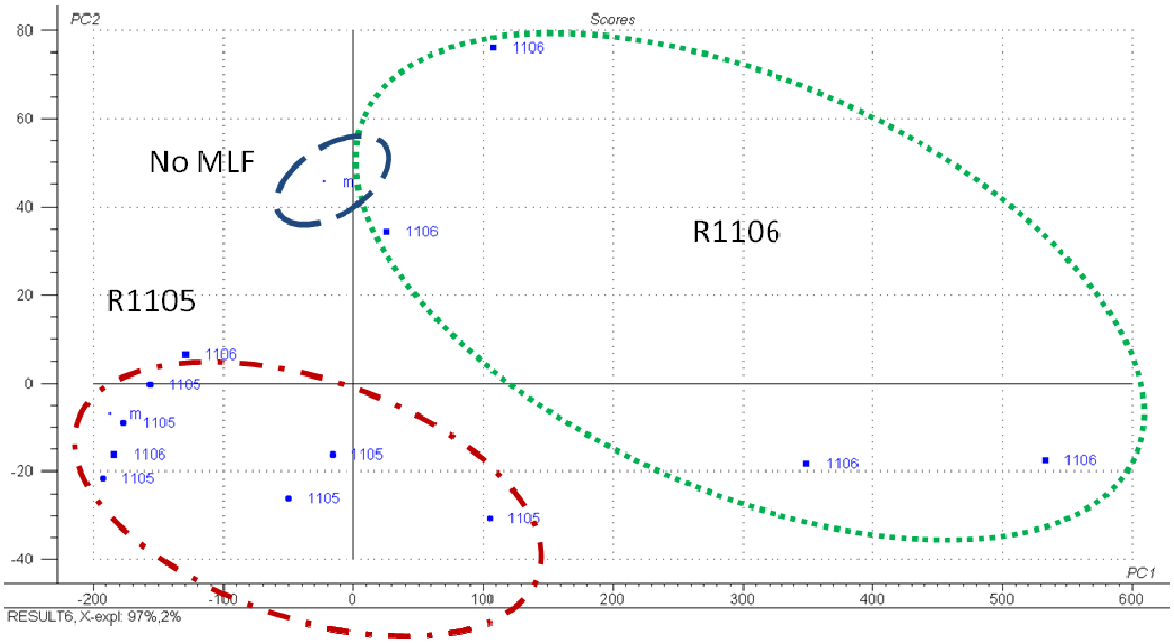

Figure 2. PCA of the fingerprints, obtained with the SmartNose® of the Riesling wines (pH 3.6) before and after MLF. PC1 and PC2 cumulatively explained 99% of the total variance.

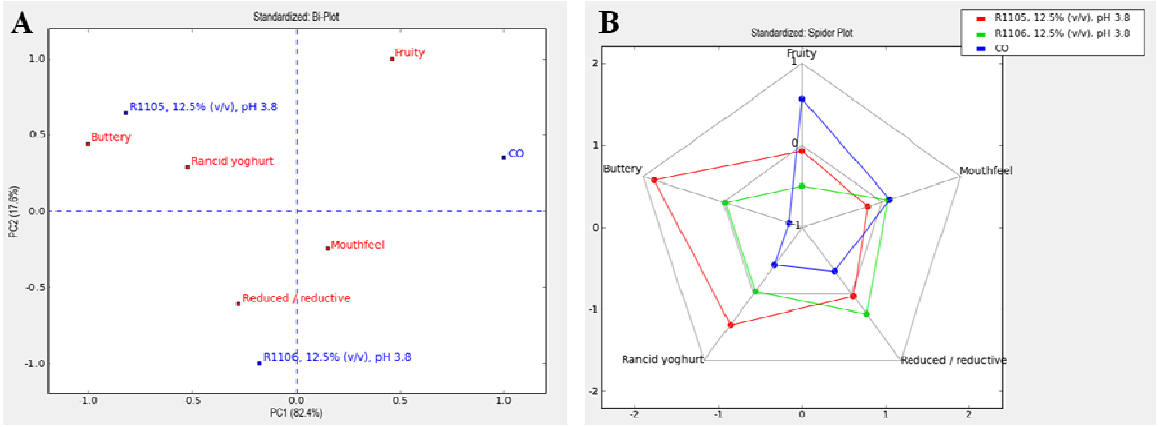

Figure 3. Standardised PCA biplot (A) and standardised spider plot (B) of the Riesling wines with pH 3.8.

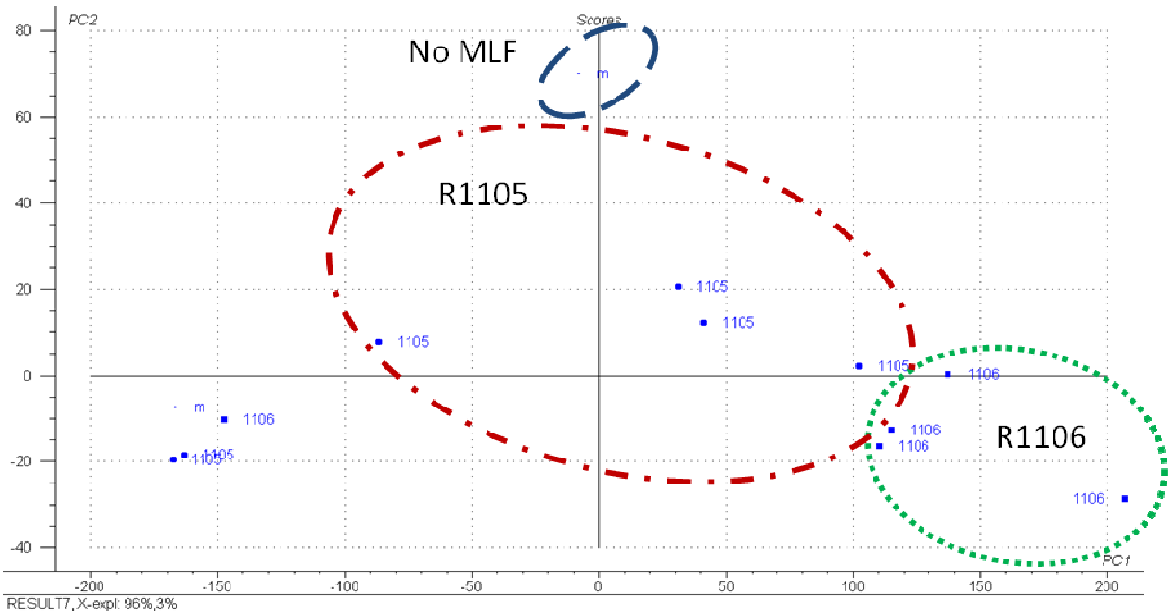

Figure 4. PCA of the fingerprints, obtained with the SmartNose®, of the Riesling wines (pH 3.8) before and after MLF. PC1 and PC2 cumulatively explained 99% of the total variance.

Chardonnay wines

Descriptive analysis results from the Chardonnay wines showed also differences among the bacterial strains tested and the control wine in terms of sensory attributes.

Figure 5 shows the standardised biplot and spider plot of the Chardonnay wines with pH 3.2. Results from the PCA clearly show differentiation between the different bacteria and the control wine for the measured sensory attributes. Three groups of samples were identified. The control sample (Co) grouped towards fruity aroma while R1106 is correlated with the mouthfeel and also buttery and reductive attributes.

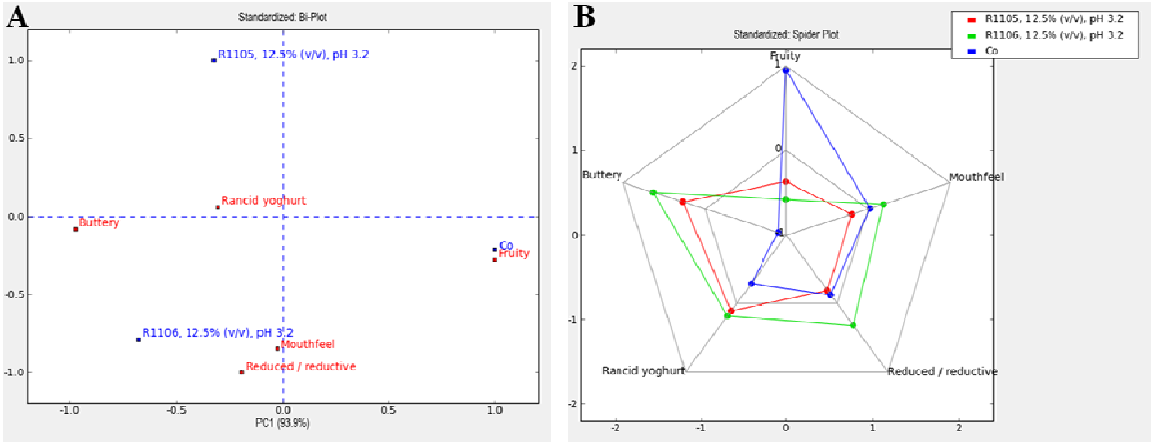

Figure 5. Standardised PCA biplot (A) and standardised spider plot (B) of the Chardonnay wines with pH 3.2.

The fingerprints of the Chardonnay wines (pH 3.2), obtained with the electronic nose illustrated that the samples could be separated according to bacterial strain and control wine which has not been inoculated with bacteria. The score plot of the first two principal components (PC1 and PC2) is shown in Figure 6.

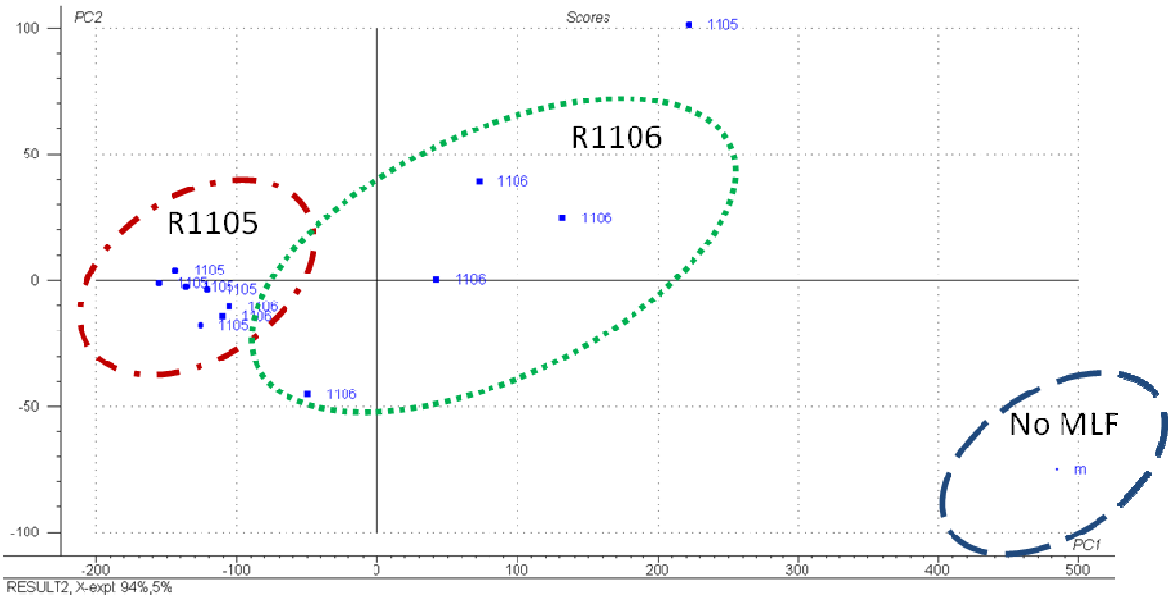

Figure 6. PCA of the fingerprints, obtained with the SmartNose® of the Chardonnay wines (pH 3.2) before and after MLF.

Figure 7 shows the average results of the descriptive intensity analysis of the Chardonnay wines with pH 3.6. The wines fermented with R1106 again clearly separated according to buttery notes and a greater mouthfeel, while the wines inoculated with R1105 in comparison, seemed to be fruitier.

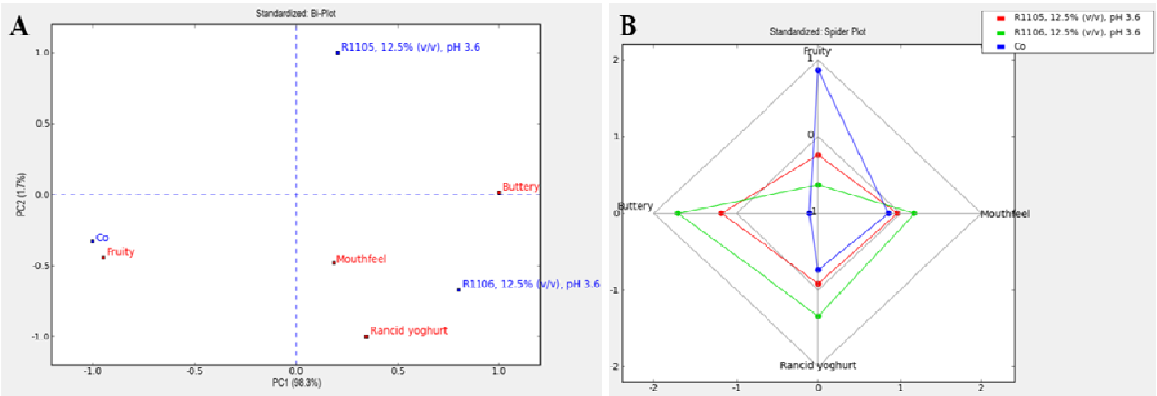

Figure 7. Standardised PCA biplot (A) and standardised spider plot (B) of the Chardonnay wines with pH 3.6.

However, the SmartNose® could not distinguish between the different treatments.

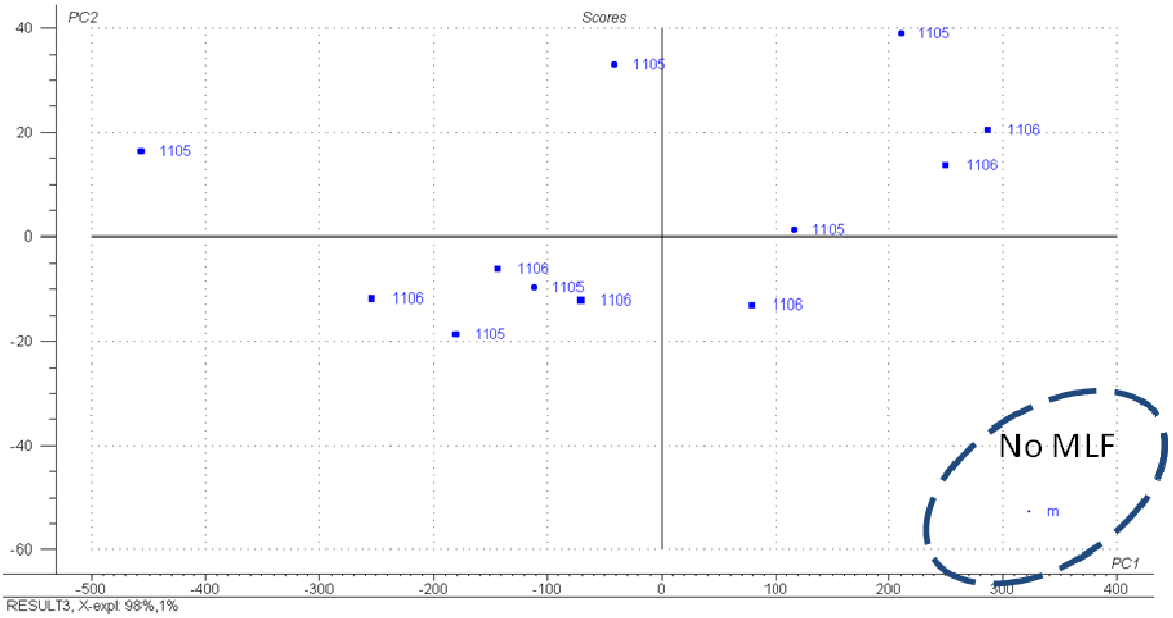

Figure 8. PCA of the fingerprints, obtained with the SmartNose® of the Chardonnay wines (pH 3.6) before and after MLF.

Figure 9 shows the average results of the descriptive intensity analysis of the Chardonnay wines with pH 3.8. The wines fermented with R1106 again separated according to mouthfeel, while the wines inoculated with R1105 seemed to have more buttery notes than in the wines at pH 3.2 and 3.6. Fingerprints obtained with the electronic nose, illustrated that the samples could be separated according to bacterial strain and control wine, (Figure 10).

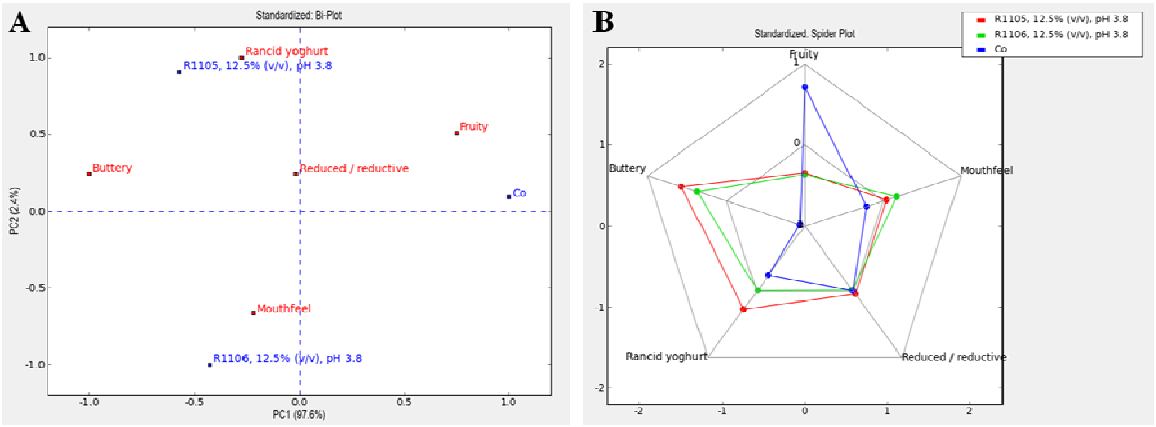

Figure 9. Standardised PCA biplot (A) and standardised spider plot (B) of the Chardonnay wines with pH 3.8.

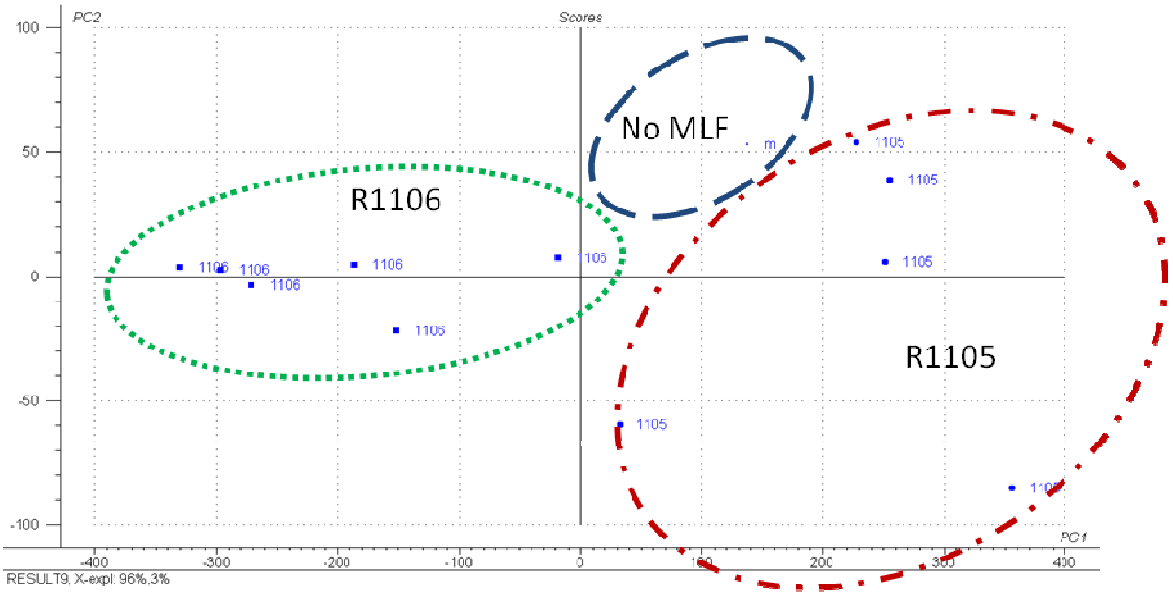

Figure 10. PCA of the fingerprints, obtained with the SmartNose®, of the Chardonnay wines (pH 3.8) before and after MLF.

Preliminary descriptive analysis of the white wines revealed sensory differences amongst the wines with and without MLF. The wines without MLF were correlated with fruitier aromas. Moreover, differences between the bacterial strains were observed. Wines fermented with *O. oeni* R1106 showed a consistent increase of mouthfeel in both cultivars. Riesling wines fermented with *O. oeni* R1105 seemed to have more intense buttery notes than the wines fermented with R1106. In contrary, in the Chardonnay wines with pH 3.2 and 3.6, this typical MLF aroma was stronger in the treatments inoculated with R1106.

Future research should also focus on more sensory evaluations with a trained panel. In addition, consumer preferences could be investigated.

A MS-based electronic nose was explored as a rapid technique for fingerprinting of volatile aroma compounds in white wines before and after MLF. Not in all cases a clear separation of the wine samples according to wine with and without MLF or to bacterial strain could be achieved.

However, the present study only used a limited number of white wines and bacterial strains, and therefore, caution must be exercised in extending the applicability of the technique to discriminate between wine samples until further work is completed. Addi-

tional studies are needed in order to improve the method specificity and accuracy and to extend the discrimination to other cultivars or bacterial starter cultures.

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9 Eidesstattliche Erklärung

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die ich wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Geisenheim,

Caroline Knoll

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