The selection and characterisation of lactic acid bacteria to be used as a mixed starter culture for malolactic fermentation

by

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Thesis presented in partial fulfilment of the requirements for the degree of
Master of Agricultural Science

at
Stellenbosch University
Department of Viticulture and Oenology, Faculty of AgriSciences

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March 2010

Declaration

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Date: 17/12/2009

Summary

The quality of wine is influenced and determined by various factors, one of which includes the process of malolactic fermentation (MLF). MLF plays an integral role in the flavour and sensory profile of most red wines as well as some white wines like Chardonnay. This process is conducted by lactic acid bacteria (LAB), specifically of the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc*. Of these, *Oenococcus oeni* is best adapted to survive in the harsh wine environment.

MLF is defined as the conversion of L-malic acid to L-lactic acid and carbon dioxide. The conversion of the dicarboxylic malic acid to the monocarboxylic lactic acid results in a decrease in acidity and an increase in pH, to give a softer mouthfeel and more favourable flavour profile. A further reason for conducting MLF in wine includes the improvement of microbial stability due to the removal of malic acid as a possible substrate for microorganisms. Recently, research focus has shifted to the ability of MLF and LAB to alter the aroma profile of wine via the production and/or modification of certain aroma compounds.

In order for wine LAB to conduct MLF, they need to be able to survive the harsh and challenging wine environment. Conditions in South African wines are particularly challenging due to the long, hot ripening seasons resulting in high sugar concentrations which give high ethanol concentrations. Some LAB also struggle to adapt to an environment with high pH and low malic acid concentrations. These factors, combined with the use of sulphur dioxide, cause LAB to struggle in conducting and completing successful MLF. Many of the commercial starter cultures that are currently available contain LAB that have not been isolated from South African wine and are therefore not optimal for use under these challenging wine conditions. *Oenococcus oeni* is also the single LAB culture present in all commercially available starter cultures.

The overriding goal of this study was to create a MLF starter culture containing a mixture of LAB cultures, namely *O. oeni* and *Lactobacillus plantarum*, which can successfully convert malic acid to lactic acid, ensure microbial stability, but also make a positive contribution to the wine aroma profile. *Lactobacillus plantarum* has previously been considered for possible use in a commercial starter culture. The LAB isolates used in this study were selected from the Institute for Wine Biotechnology culture collection as well as isolated from spontaneous MLF.

The first objective was to characterise these LAB strains for important traits and for possible use as a MLF starter culture. A total of 23 strains were identified as *O. oeni* and 19 strains as *Lb. plantarum*. The identified strains were screened in a synthetic wine medium for their ability to convert malic acid to lactic acid. Based on the LAB strain performance in the synthetic wine medium, seven strains of both *O. oeni* and *Lb. plantarum* were selected. These 14 strains were screened for the presence of genes encoding for enzymes responsible for biogenic amine production and were found to contain none of the genes associated with the formation of histamine, tyramine or putrescine. The LAB strains were genetically screened for enzymes

associated with aroma modification by LAB during MLF. The enzymes of interest that were screened for included *β*-glucosidase, esterase, protease and phenolic acid decarboxylase (PAD). The *Lb. plantarum* strains were found to possess more diverse enzymatic profiles related to aroma than *O. oeni*. The biggest differences were observed for the presence of *β*-glucosidase and PAD.

The second objective was to perform small-scale fermentations with the individual LAB isolates. The individual isolates were evaluated in Pinotage and based on these results; three strains of each *O. oeni* and *Lb. plantarum* were selected for evaluation in mixed culture fermentations. The mixed cultures were evaluated in Pinotage, Shiraz and Cabernet Sauvignon in the 2008 vintage. As a third objective, the wines were also analytically and sensorially evaluated to investigate the changes in the aroma profile that could be attributed to the presence of the mixed LAB isolates. Based on the fermentation data as well as data pertaining to the aroma modification, three mixed cultures were selected for evaluation in the 2009 vintage in Pinotage, Cabernet Sauvignon and Chardonnay. The mixed cultures were able to successfully complete MLF in fermentation periods comparable to that of a commercial culture used as control. The different LAB cultures had distinct and diverse effects on the wine aroma profile. The *O. oeni* strain played a larger role in the ester concentration present after MLF, while the *Lb. plantarum* strain had a larger effect on the higher alcohol and volatile fatty acid concentration upon completion of MLF.

The results generated by this novel study clearly indicate the potential of a mixed LAB starter culture for conducting MLF. The mixed cultures successfully completed MLF and made a positive contribution to the wine aroma profile.

Opsomming

Die kwaliteit van wyn word beïnvloed en bepaal deur verskeie faktore en wynbereidings prosesse, wat die proses van appelmelksuurgisting (AMG) insluit. AMG speel 'n integrale rol in die sensoriese profiel van meeste rooiwyne, sowel as sommige witwyne soos Chardonnay.

AMG word gedefinieër as die omskakeling van L-appelsuur na L-melksuur en koolstofdioksied. Hierdie omskakeling kan toegeskryf word aan die teenwoordigheid van melksuurbakterieë (MSB), spesifiek spesies van die genera *Oenococcus, Lactobacillus*, *Pediococcus* en *Leuconostoc*. Vanuit hierdie wyn MSB, is *Oenococcus oeni* die spesies wat die beste aanpas en oorleef onder stresvolle wyn kondisies. Die omskakeling van appelsuur, 'n dikarboksielsuur, na melksuur, 'n monokarboksielsuur, lei tot 'n vermindering in suurheid en 'n verhoging in pH. Hierdie vermindering in suurheid gee 'n sagter en meer geronde mondgevoel aan die wyn en dra by tot 'n meer aangename geurprofiel. 'n Verdere rede vir AMG in wyn is om mikrobiese stabiliteit te verseker deurdat appelsuur verwyder word as 'n moontlike koolstof substraat vir mikroörganismes. Onlangs het navorsing begin fokus op AMG en die vermoë van MSB om die aroma profiel van wyn te beïnvloed deur die produksie/modifisering van sekere aroma komponente.

Vir MSB om AMG te kan deurvoer, moet hulle kan oorleef in die stresvolle wynomgewing. Wyntoestande in Suid-Afrika is veral uitdagend vir die oorlewing van mikroörganismes as gevolg van lang, warm somers wat lei tot 'n matriks met 'n hoë suikerkonsentrasie en wyn met 'n hoë etanolkonsentrasie. 'n Omgewing met 'n hoë pH en lae appelsuur konsentrasie, kan ook bydrae tot stresvolle kondisies vir MSB. Hierdie parameters, tesame met die gebruik van swaweldioksied, maak dit moeilik vir MSB om AMG te inisieer en te voltooi. Sommige van die kommersiële aanvangskulture wat tans beskikbaar is, bevat nie MSB wat onder Suid-Afrikaanse wyntoestande geïsoleer is nie en daarom is dit nie altyd optimaal vir gebruik nie. *Oenococcus oeni* is ook die enkele MSB kultuur wat in alle kommersiële kulture gebruik word.

Die hoofdoelwit van hierdie studie was om 'n potensiële kommersiële aanvangskultuur te ontwikkel wat 'n mengsel van MSB bevat. Hierdie aanvangskultuur moet AMG suksesvol kan voltooi, mikrobiologiese stabiliteit bevorder en steeds die wynaroma positief kan beïnvloed. Bakterierasse van *O. oeni* en *Lb. plantarum* is geselekteer vir gebruik in hierdie studie. *Lactobacillus plantarum* het reeds in vorige studies potensiaal getoon as 'n moontlike aanvangskultuur. Die MSB isolate vir hierdie studie is geselekteer uit die Instituut vir Wynbiotegnologie se kultuurversameling en geïsoleer uit spontane AMG fermentasies.

Die eerste doelwit was om hierdie MSB isolate te karakteriseer vir belangrike eienskappe en die moontlike gebruik as 'n kommersiële AMG aanvangskultuur. 'n Totaal van 23 *O. oeni* en 19 *Lb. plantarum* isolate is geïdentifiseer. Hierdie isolate is in 'n sintetiese wynmedium geëvalueer vir hul vermoë om appelsuur na melksuur om te skakel. Op grond van hul reaksie in die sintetiese wynmedium, is sewe isolate van elk van die *O. oeni* en *Lb. plantarum* geselekteer. Hierdie 14 isolate is ondersoek vir die teenwoordigheid van die gene wat kodeer vir biogeenamien produksie

en daar is gevind dat geen van die isolate enige van die biogeenamien gene wat ondersoek is, naamlik histamien, tiramien en putresien besit nie. Die MSB isolate is geneties ondersoek vir die teenwoordigheid van dié gene wat kodeer vir ensieme wat die aromaprofiel tydens AMG beïnvloed. Dié ensieme sluit *β*-glukosidase, esterase, protease, fenoliese suurdekarboksilase en sitraatliase in. Daar is gevind dat die *Lb. plantarum* isolate meer diverse ensiemprofiele as *O. oeni* besit. Die grootste verskille in die ensiemprofiele kan toegeskryf word aan die teenwoordigheid van *β*-glukosidase en fenoliese suurdekarboksilase.

Die tweede doelwit was om kleinskaalse AMG fermentasies met die individuele MSB isolate uit te voer. Die individuele isolate is in Pinotage geëvalueer. Volgens hierdie resultate is drie isolate van elk van die *O. oeni* en *Lb. plantarum* geselekteer om in gemengde kulture getoets te word. Die gemengde kulture is in Pinotage, Shiraz en Cabernet Sauvignon in 2008 geëvalueer. As ’n derde doelwit is hierdie wyne ook analities en sensories geëvalueer om die veranderinge in die aromaprofiele as gevolg van die teenwoordigheid van die MSB te ondersoek. Op grond van die fermentasiedata, sowel as die data oor die aromaveranderinge, is drie gemengde kulture geselekteer vir evaluering in Pinotage, Cabernet Sauvignon en Chardonnay in 2009. Die gemengde kulture kon AMG suksesvol voltooi met fermentasietempo’s wat vergelykbaar was met dié van ‘n kommersiële AMG kultuur wat as kontrole gebruik is. Die verskillende MSB kulture het spesifieke en uiteenlopende uitwerkings op die wynaroma gehad. Die *O. oeni* isolaat in die gemengde kultuur blyk ‘n belangriker rol te speel in die esterkonsentrasie na AMG, terwyl die *Lb. plantarum* isolaat ’n groter effek het op die hoër alkohol en vlugtige vetsuurinhoud na AMG.

Die resultate wat deur hierdie unieke studie gegenereer is, gee ’n aanduiding van die potensiaal van ’n gemengde MSB aanvangskultuur vir AMG. Die gemengde kulture kon AMG suksesvol voltooi en ‘n positiewe bydrae tot die aromaprofiel van die wyn lewer.

This thesis is dedicated to my family

Hierdie tesis is opgedra aan my familie

Biographical sketch

Elda Lerm was born on 23 July 1985 in Cape Town and matriculated at Durbanville High School in 2003. Elda obtained her BScAgric degree *cum laude* (Oenology Specialised) at Stellenbosch University in 2007. In 2008, she enrolled at the same University for a Masters degree in Oenology.

Acknowledgements

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

- **Prof. M. du Toit**, (Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University) who acted as supervisor and provided unending support, motivation and encouragement, for allowing me the opportunity to be one of her students and for the critical evaluation of this manuscript;

- **Lynn Engelbrecht**, (Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University) for being such a patient teacher, for her endless support and friendship and for the critical evaluation of this manuscript;

- **Fellow colleagues**, (Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University) for providing valuable training, advice and assistance in the laboratory;

- **Anchor Yeast, The National Research Foundation and Postgraduate Merit Bursary** for financial support;

- **Cellar staff**, (Experimental Cellar, Department of Viticulture and Oenology, Stellenbosch University) for support, assistance and willingness to help in the cellar;

- **My parents and sister**, Pieter, Lynette and Adél and my grandparents, Johan and Elsa Lerm for never failing to believe in me and for their constant support, encouragement and love;

- **Sulette Malherbe, Caroline Knoll, Anita Smit, Anita Burger, Samantha Fairbairn and Nicolet Bedeker**, for their support and friendship through many late nights; and

- **God Almighty**, for blessing me with the ability to do what I love.

Preface

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of the South African Journal of Enology and Viticulture.

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Malolactic fermentation: A Review

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Chapter 1

General introduction and project aims

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

There are two main fermentation processes that take place during vinification. Alcoholic fermentation (AF) is the primary fermentation conducted by the yeast, *Saccharomyces cerevisiae*. Malolactic fermentation (MLF) is the secondary fermentation process that usually follows upon the completion of AF but may also occur concurrently. During this process L-malic acid is reduced to L-lactic acid and carbon dioxide and this reaction is catalysed by the malolactic enzyme (Davis *et al.,* 1985; Lonvaud-Funel, 1995). Lactic acid bacteria (LAB) are responsible for this step in the winemaking process, especially species from the genera *Leuconostoc, Pediococcus, Lactobacillus*, as well as *Oenococcus oeni* (formerly known as *Leuconostoc oenos;* Dicks *et al.,* 1995) (Liu, 2002).

In wine, MLF is performed for three main reasons. Firstly, the conversion of the dicarboxylic malic acid to the monocarboxylic lactic acid results in a reduction in the acid concentration with a concomitant increase in the pH. Secondly, the removal of lactic acid as a possible substrate for further metabolic reactions contributes to the microbial stability of the wine. Lastly, MLF has a profound effect on the wine aroma profile and the metabolism of the LAB will alter the eventual sensorial perception of the wine (Davis *et al.,* 1988; Kunkee, 1991; Maicas *et al.*, 1999; Liu, 2002; Ugliano *et al.,* 2003; Swiegers *et al*., 2005). Acid reduction is a more important consideration in countries in the cooler climate regions. In these countries, too high acid levels are problematic due to lower temperatures, whereas in South Africa, winemakers struggle to retain high acid levels due to the long, hot summers.

In South Africa, these higher temperatures during the ripening period lead to the production of grapes with a high sugar content and lower acid concentrations. Concomitantly, winemakers struggle with wines that have a high pH, require the use of high levels of sulphur dioxide (SO_2), and have high ethanol content. *Oenococcus oeni* has best adapted to this harsh wine environment and is therefore the LAB selected for use in commercial MLF starter cultures. Some *Lactobacillus* species have also shown promise in surviving under wine conditions (Wibowo *et al*., 1985; Davis *et al.,* 1988; Drici-Cachon *et al.,* 1996; Lonvaud-Funel, 1999; G-Alegría *et al.,* 2004; Pozo-Bayón *et al.,* 2005). *Lactobacillus plantarum* has shown the most promise for use as a starter culture and also has a more complex enzymatic profile than *O. oeni,* specifically with regards to *β*-glucosidase, which could play an important role in the modification of the sensorial profile of the wine (Guerzoni *et al*., 1995; Pozo-Bayón *et al.,* 2005; Swiegers *et al.,* 2005; Matthews *et al.,* 2006; Mtshali *et al*., 2009). LAB are able to modify wine aroma and flavour by metabolising grape constituents, modifying grape- or yeast-derived secondary metabolites and by adsorbing flavour compounds to the cell wall (Bartowsky and Henschke, 1995). Positive aroma compounds of interest include diacetyl and 2,3-butanediol, esters (Liu, 2002) and higher alcohols, as well as compounds with

negative organoleptic qualities such as volatile sulphur compounds, acetic acid and volatile phenols (Swiegers *et al.,* 2005). Inoculation with a commercial starter culture could be beneficial in reducing or eliminating the risks associated with uncontrolled or spontaneous MLF. These include wine spoilage via the production of aroma compounds that contribute to off-flavours (acetic acid, mousiness and volatile phenols) as well as health-impacting compounds like biogenic amines and ethyl carbamate (Chatonnet *et al*., 1999; Costello *et al*., 2001; Lonvaud-Funel, 2001).

The changes associated with MLF and the metabolism of LAB are largely dependant on the selected strain of LAB and therefore the selection, screening and characterisation of isolates for use in a starter culture are essential (Britz and Tracey, 1990; Henick-Kling, 1993). There are various important criteria to consider when selecting cultures for possible use in a MLF starter culture. These include the following: the ability to tolerate high ethanol and SO_2 concentrations, low pH, good growth characteristics under winemaking conditions, compatibility with the selected yeast strain, the inability to produce biogenic amines and the lack of off-flavour or off-odour production (Wibowo *et al*., 1985; Kunkee, 1991; Fugelsang and Zoecklein, 1993; Henick-Kling, 1993; Le Jeune *et al*., 1995; Drici-Cachon *et al*., 1996; Lonvaud-Funel, 2001; Marcobal *et al*., 2004; Volschenk *et al*., 2006).

It is essential to evaluate the influence of various factors on the selected and screened cultures. Factors including ethanol, pH, temperature and SO_2, will have a direct effect on the ability of the LAB culture to survive in the wine environment and complete MLF (Kunkee, 1991; Vaillant *et al*., 1995). Small-scale vinifications therefore play an integral role in evaluating possible cultures under winemaking conditions (Bou and Powell, 2006). These influencing factors do not only affect the growth ability and the malolactic activity of LAB, but also influence the effect that the LAB cultures will have on the wine aroma. An additional area of research to explore is the impact of different inoculation times on bacterial performance during MLF. It is also important to investigate the effect of different inoculation times on the aroma contribution of the different LAB cultures during MLF.

There are currently very few MLF starter cultures that are optimal for use under South African wine conditions and studies done by various authors like Guerzoni *et al.* (1995), Hernández *et al.* (2007) and G-Alegría *et al.* (2004), all focus on the individual performance of *Lb. plantarum* and *O. oeni* during MLF. None of the currently available MLF starter cultures contain different genera of LAB that could possibly have a more positive and pronounced effect on the wine aroma.

1.2 PROJECT AIMS

This study forms an integral part of a larger research programme on MLF that is being conducted at the Institute for Wine Biotechnology. The main aim of the programme is evaluating natural LAB isolated from the South Africa wine industry as potential MLF starter cultures. The LAB isolates of

interest are *O. oeni* and *Lb. plantarum* species. The principal objective of this study was to assess using *O. oeni* and *Lb. plantarum* in mixed starter cultures for conducting MLF.

The specific aims and approaches of this study were as follow:

(i) to characterise wine LAB for possible use in a MLF starter culture by evaluating their ability to degrade malic acid in a synthetic wine medium; screening for the absence of genes encoding for biogenic amine production; the genetic screening of enzymes important in wine aroma production including *β*-glucosidase, protease, esterase, citrate lyase and phenolic acid decarboxylase;

(ii) to assess all selected *O. oeni* and *Lb. plantarum* strains as single cultures in Pinotage with regard to their ability to degrade malic acid;

(iii) to select and evaluate three *O. oeni* and *Lb. plantarum* strains in different combinations in 2008 by inoculating wines after AF;

(iv) to evaluate the three best combinations in 2009 in three cultivars using co-inoculation and sequential inoculation to assess malic acid degradation rate;

(v) to determine the volatile aroma- and carbonyl compounds produced during MLF using analytical techniques; and

(vi) to do multivariate data analysis on all data sets generated.

To our knowledge, this is the first study on mixed MLF starter cultures.

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Chapter 2

Literature Review

Malolactic fermentation: A Review

This manuscript will be submitted for publication in

South African Journal of Enology and Viticulture

2. LITERATURE REVIEW

Malolactic fermentation: A Review

2.1 INTRODUCTION

Malolactic fermentation (MLF) is an intricate process that usually follows after the completion of the alcoholic fermentation (AF) by yeasts. Although MLF is regarded as a secondary fermentation process, it plays an integral role in the production of the majority of red wines, as well as some white cultivars including Chardonnay and some sparkling wines.

There are three main reasons for conducting MLF in wine. Firstly, the deacidification of the wine with a concomitant increase in pH, secondly, to contribute to the microbial stability by the removal of malic acid as a possible substrate and thirdly, the modification of the wine aroma profile (Davis *et al.,* 1988; Kunkee, 1991; Maicas *et al*., 1999; Liu, 2002; Ugliano *et al.,* 2003). In cooler climate countries the deacidification process is regarded as the most important modification associated with MLF, while the change in the sensory profile of the wine is a more important consideration in countries where deacidification is of less significance, i.e. warmer regions where lower concentrations of malic acid are present in the grapes.

The MLF reaction is defined as the conversion of L-malic acid, a dicarboxylic acid, to L-lactic acid, a monocarboxylic acid, with the production of carbon dioxide (CO_2). The reaction is catalysed by lactic acid bacteria (LAB), including bacteria from the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Wibowo *et al.,* 1985). Of these, *Oenococcus oeni* is best adapted to the harsh wine environment, including conditions of high alcohol, low pH and the presence of sulphur dioxide (SO_2) (Wibowo *et al*., 1985; Davis *et al.,* 1988; Drici-Cachon *et al.,* 1996; Lonvaud-Funel, 1999). Various review articles on MLF have appeared over the years (Wibowo *et al*., 1985; Davis *et al.,* 1988; Kunkee, 1991), with increasing amounts of information being generated regarding this important step in the winemaking process as well as the characterisation of the microorganisms involved. Some of the most recent review articles include Lonvaud-Funel (1999) and Liu (2002), with the focus falling on the metabolism of wine associated LAB, specifically *O. oeni*. In addition to the information being generated on the metabolic processes associated with wine LAB, the molecular aspects of LAB are also being investigated. At the beginning of the 21st century, the DOE Joint Genome Institute commenced the sequencing of the entire genome of *O. oeni* PSU-1, a strain isolated by Beelman *et al*. (1977) (Bartowsky, 2005). The genome is now fully sequenced which allows for more intensive studies regarding the physiology, genetic diversity and performance of *O. oeni* starter cultures.

The focus of this literature review will be to summarise key aspects associated with the process of MLF. The MLF reaction as well as the main LAB found in wine will be discussed. The use of commercial starter cultures and the influence of different inoculation times are considered.

Various factors influence this fermentation process, such as wine parameters, microorganisms and compounds originating from the grapes, and will also be discussed. As recent research focus has fallen on the organoleptic changes in wine undergoing MLF, the important aroma compounds responsible for MLF aroma characteristics are critically reviewed. The final section of the review will highlight some practical considerations for the monitoring of MLF to ensure the successful completion of MLF with a positive contribution to the aroma profile.

2.2 MALOLACTIC FERMENTATION

LAB possess three possible enzymatic pathways for the conversion of L-malic acid to L-lactic acid and CO_2. The first is the direct conversion of malic acid to lactic acid via malate decarboxylase, also known as the malolactic enzyme (MLE). This reaction requires NAD^+ and Mn^{2+} as cofactors and no free intermediates are produced during this decarboxylation reaction. The rate of malate decarboxylation by LAB is correlated to the specific malolactic activity of the bacterial cell (Bartowsky, 2005). The main wine LAB utilise this pathway to generate lactic acid. A paper written by Lonvaud-Funel (1995) highlighted the main features of the malate decarboxylase (*mleA*) gene. The enzyme has been purified from various LAB species that were isolated from wines and grapes, including species from *Lactobacillus* and *Leuconostoc* (Lonvaud-Funel, 1995). The second pathway utilises the malic enzyme to convert L-malic acid to pyruvic acid, which is subsequently reduced by L-lactate dehydrogenase to lactic acid. The third possible pathway is the reduction of malate by malate dehydrogenase to oxaloacetate, followed by decarboxylation to pyruvate and reduction to lactic acid (Lonvaud-Funel, 1999).

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

2.3 LACTIC ACID BACTERIA ASSOCIATED WITH WINE

LAB are coccoid to elongated cocci or rod-shaped bacilli, Gram-positive, non-sporing and non-respiring bacteria. As the name suggests, lactic acid is the major product formed during the fermentation of carbohydrates. LAB species from the genera *Leuconostoc, Pediococcus,*

Lactobacillus as well as *O. oeni*, are accountable for the changes to the wine matrix during the fermentation process (Wibowo *et al.,* 1985). *Oenococcus oeni* has best adapted to the wine environment and concomitantly the majority of LAB present in wine belong to this species. *Oenococcus oeni* strains are also the selected bacteria used for commercial starter cultures (Wibowo *et al*., 1985; Davis *et al.,* 1988; Drici-Cachon *et al.,* 1996; Lonvaud-Funel, 1999).

2.3.1 EVOLUTION OF LACTIC ACID BACTERIA POPULATION

The evolution of LAB from the vineyard to the final vinification stages have been documented, but show considerable variability due to region, cultivar and vinification procedures. It is clear that there is a successional growth of several species of LAB during vinification (Wibowo *et al*., 1985; Boulton *et al*., 1996; Fugelsang and Edwards, 2007). *Oenococcus oeni* is the main LAB species associated with wine; *Pediococcus damnosus*, *Pediococcus parvulus* and *Pediococcus pentosaceus* mostly occur after MLF and in higher pH wines and several *Lactobacillus* species also occur after MLF (Wibowo *et al*., 1985; Powell *et al*., 2006).

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

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

After the completion of AF and the bacterial lag phase, the surviving bacterial cells, most commonly *O. oeni*, start to multiply. This phase is characterised by vigorous bacterial growth and the start of MLF is induced when bacterial populations reach 10^6 to 10^8 cfu/mL (Wibowo *et al*., 1985; Lonvaud-Funel, 1999). The pH of the wine is imperative in determining which species of LAB

are present, with values above pH 3.5 favouring the growth of *Lactobacillus* and *Pediococcus* species, whereas the *O. oeni* population tend to dominate at lower pH values (Davis *et al*., 1986b; Henick-Kling, 1993).

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

By understanding the evolution of LAB from the grape and through the different vinification procedures as well as their metabolic requirements, it is possible to control what species of LAB occur at a particular stage and ensure positive contributions by the LAB during MLF.

2.3.2 METABOLISM OF LACTIC ACID BACTERIA

2.3.2.1 Metabolism of carbohydrates

LAB possess two main pathways for the metabolism of glucose and a single pathway for the metabolism of pentose sugars. The two pathways for the metabolism of glucose include the glycolysis/Embden-Meyerhof-Parnas (EMP) pathway and the 6 phosphogluconate/ phosphoketolase (6-PG/PK) pathway (Fugelsang and Edwards, 1997).

Glucose, as a free sugar, is transported into the cell where it is phosphorylated by hexokinase, a reaction which is ATP-dependant, before it enters one of the two mentioned pathways. The EMP pathway, also known as homolactic fermentation in LAB, leads to the formation of lactic acid as the main end-product, as well as the production of CO_2. This pathway is utilised by *Pediococcus* strains and the metabolism of one mole of glucose produce two moles of lactic acid as well as a net amount of two ATP. The 6-PG/PK pathway, also known as heterolactic fermentation, result in the production of lactic acid and CO_2, as well as the end-products ethanol and acetate. Species of LAB that make use of this pathway include all the strains of *Leuconostoc*, some *Lactobacillus* strains and *O. oeni*. One mole of glucose metabolised via this pathway will lead to the formation of equimolar amounts of each of lactic acid, ethanol and CO_2, as well as one mole of ATP (Fugelsang and Edwards, 1997).

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

According to the pathway used for the metabolism of carbohydrates, LAB can be divided into three metabolic groups. Each group also differ according to the enzymes that are needed for carbohydrate metabolism. The obligatory homofermentors only make use of the EMP pathway for

carbohydrate metabolism. They possess the aldolase enzyme but the phosphoketolase enzyme is absent. All wine *Pediococcus* species are included in this group. The obligatory heterofermentors include *Lactobacillus brevis*, *Lactobacillus hilgardii*, *Leuconostoc* species and *O. oeni*. This group utilise the 6-PG/PK fermentation pathway for the metabolism of carbohydrates. This group displays phosphoketolase activity but do not possess the aldolase enzyme. Some *Lactobacillus* species are facultative heterofermentors. These include *Lb. casei* and *Lb. plantarum*. These LAB make use of the EMP pathway for hexose metabolism and the 6-PG/PK pathway for the metabolism of pentose sugars and other substrates. These LAB only possess the aldolase enzyme (Fugelsang and Edwards, 1997).

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

2.4 COMMERCIAL STARTER CULTURES AND TIMING OF INOCULATION

2.4.1 COMMERCIAL STARTER CULTURES

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

MLF starter cultures were available in liquid form and used for decades until the early 1980s. At that time, frozen and freeze-dried LAB starter cultures were developed. Shortly after, in the 1990s, direct inoculation freeze-dried starter cultures were developed. Their use has made it easier to control and predict the progression of MLF in wine (Specht, 2006). These commercial cultures are also easy to ship, store and use, which adds to their increasing popularity. A commercial starter culture contains a very high population of viable bacteria, ± 10^{11} cfu/g, to ensure that any loss in viability due to the wine conditions are not detrimental to the completion of MLF (Henick-Kling, 1993; 1995). **Table 2.1** lists some of the commercial MLF starter cultures that are available today.

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

Table 2.1 MLF starter cultures that are available as well as their main characteristics and applications (compiled from company websites).

Name	Company	Characteristics	Application	Form
Viniflora CH16	Chr. Hansen	Temperature: 17-25°C Alcohol tolerance:16% pH: 3.4 TSO_2* tolerance: 40 ppm	High alcohol red and some types of rosé wines	Frozen/Freeze-dried
Viniflora CH35	Chr. Hansen	Temperature: 15-25°C Alcohol tolerance: 14% pH: 3.1 TSO_2 tolerance: 45 ppm	White and some rosé wines	Frozen/Freeze-dried
Viniflora CH11	Chr. Hansen	Temperature: 14-25°C Alcohol tolerance: 15% pH: 3 TSO_2 tolerance: 35 ppm	Low pH white and some rosé wines	Frozen/Freeze-dried
Viniflora oenos	Chr. Hansen	Temperature: 17-25°C Alcohol tolerance: 14% pH: 3.2 TSO_2 tolerance: 40 ppm	Red, rosé and white wines	Frozen/Freeze-dried
Viniflora Ciné	Chr. Hansen	Temperature: 17-25°C Alcohol tolerance: 14% pH: 3.2 TSO_2 tolerance: 30 ppm	Red, rosé and white wines, sparkling wine with no diacetyl production	Frozen
Biolact Acclimatée	AEB Group	NA**	NA	Freeze-dried
Biolact Acclimatée BM	AEB Group	Temperature: 12°C pH: 3	NA	Freeze-dried
Biolact Acclimatée PB1025	AEB Group	Temperature: 15-18°C Alcohol tolerance: high pH: 2.9 TSO_2 tolerance: high	White, rosé and young red wines	Freeze-dried
Biolact Acclimatée 4R	AEB Group	Temperature: resistance to low temp. Alcohol tolerance: high	Red wines with high tannin concentrations	Freeze-dried

Table 2.1 continued

Lactoenos B16 Standard	Laffort	Temperature: >16°C Alcohol tolerance: 16% pH: >2.9 TSO_2 tolerance: 60 ppm	Acidic white wines	NA
Lactoenos SB3 Instant	Laffort	Temperature: >16°C Alcohol tolerance: 15% pH: >3.3 TSO_2 tolerance: 30 ppm	High quality wines (undergoing barrel MLF)	NA
Lactoenos 350 PreAc	Laffort	Temperature: >15°C Alcohol tolerance: 16% pH: >3 TSO_2 tolerance: 60 ppm	Low pH white and certain rosé wines	NA
Lactoenos 450 PreAc	Laffort	Temperature: >16°C Alcohol tolerance: 17% pH: >3.3 TSO_2 tolerance: 80 ppm	Red and white wines	NA
1 Step Alpha	Lallemand	Temperature: 14°C Alcohol tolerance: high pH: > 3.3 TSO_2 tolerance: < 40 ppm	Red and white wines	Freeze-dried
1 Step VP41	Lallemand	Temperature: 17°C Alcohol tolerance: high TSO_2 tolerance: < 60 ppm	High alcohol red wines	Freeze-dried
Enoferm Alpha	Lallemand	Temperature: 14°C Alcohol tolerance: high pH: > 3.2 TSO_2 tolerance: < 50 ppm	Red and white wines	Freeze-dried
Enoferm Beta	Lallemand	Temperature: 14°C Alcohol tolerance:15% pH: > 3.2 TSO_2 tolerance: < 60 ppm	Red wines	Freeze-dried
Lalvin 31	Lallemand	Temperature: 13°C pH: > 3.1 TSO_2 tolerance: < 45 ppm	Red and white wines	Freeze-dried
Lalvin Elios 1	Lallemand	Temperature: 18°C Alcohol tolerance: high pH: > 3.4 TSO_2 tolerance: < 50 ppm	Red wine	Freeze-dried
Lalvin ICV Elios Blanc	Lallemand	pH: <3.4	White and rosé wines with difficult pH and temperature conditions	Freeze-dried

Table 2.1 continued

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

* Total SO_2

** NA: not available

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

In an effort to be more cost-effective, a technique referred to as stretching can be implemented. The stretching of starter cultures imply using less than the recommended dosage, but can also imply the re-use of commercial starter cultures as in the case of mother tank inoculation as well as inoculation from the lees of wines that have finished MLF. These are risky practices. There is a possibility of the development of spoilage microorganisms due to the decreased population of inoculated bacteria and MLF may not be successfully completed. Further risks include a lack of control over the MLF process as well as the contamination of further

fermentation vessels from a contaminated mother tank (Van der Merwe, 2007). Due to the risks associated with spontaneous or uncontrolled MLF and stretching, it is important for the winemaker to realise the benefits associated with inoculating for MLF with a starter culture as well as inoculating according to the directions of the manufacturer.

The selection and characterisation of strains for possible use in a commercial culture is crucial, due to the fact that *O. oeni* strains differ in their fermentation capabilities and growth characteristics (Britz and Tracey, 1990; Henick-Kling, 1993). Strict criteria are used for the selection of bacteria to be used as starter cultures (Davis *et al*., 1985; Vaillant *et al*., 1995; Volschenk *et al*., 2006; Krieger-Weber, 2009). These criteria include the following: tolerance to low pH, high ethanol and SO_2 concentrations, good growth characteristics under winemaking conditions, compatibility with *Saccharomyces cerevisiae*, ability to survive the production process, the inability to produce biogenic amines, the lack of off-flavour or off-odour production as well as the production of aroma compounds that could potentially contribute to a favourable wine aroma profile (Wibowo *et al*., 1985; Kunkee, 1991; Fugelsang and Zoecklein, 1993; Henick-Kling, 1993; Le Jeune *et al*., 1995; Drici-Cachon *et al*., 1996; Lonvaud-Funel, 2001; Marcobal *et al*., 2004; Volschenk *et al*., 2006). The technological and qualitative properties important in the selection criteria for LAB strains for use in starter cultures for MLF were recently summarised by Krieger-Weber (2009).

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

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

2.4.2 TIMING OF INOCULATION

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

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

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

Inoculation during AF is not a common practice and Rosi *et al*. (2003) reported the strongest antagonism between yeast and bacteria with inoculation of LAB during AF. Bacterial populations showed drastic decreases with this type of inoculation and this could be attributed to various factors including the removal of nutrients by the yeast, accumulation of SO_2, ethanol production, toxic metabolite production by the yeast and acid production by the yeast that decrease the pH. The same study found that at the end of AF, yeast presence favoured the growth and malolactic activity of LAB. This could be attributed to yeast autolysis that release vitamins, amino acids,

proteins and polysaccharides that stimulate bacterial metabolism (Henick-Kling, 1993). Early results by some authors advocate sequential inoculation as a means to avoid the problems associated with early inoculation (Ribéreau-Gayon, 1985; Henick-Kling, 1993). The advantages of sequential inoculation include the lack of adverse interactions between yeast and bacteria as well as a reduced risk of acetic acid production due to smaller residual sugar concentrations (Costello, 2006). In spite of these advantages, there are still risks related with sequential inoculation and a loss in viability may possibly be attributed to the presence of high ethanol concentrations, low pH, SO_2, other antimicrobial compounds produced by the yeast as well as nutrient depletion (Larsen *et al*., 2003).

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

2.5 FACTORS INFLUENCING MALOLACTIC FERMENTATION

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

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

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

2.5.1 YEAST-BACTERIA INTERACTIONS

A factor that the winemaker has the most control over is the selection of the yeast and bacterial culture for AF and MLF, respectively. The interaction between bacteria and yeast during AF and/or MLF will have a direct effect on LAB growth and malolactic activity. Various studies have been done to attempt an understanding of the interaction between yeast and bacteria (Henick-Kling and Park, 1994; Rosi *et al*., 2003; Arnink and Henick-Kling, 2005; Guilloux-Benatier *et al*., 2006; Jussier *et al*., 2006; Osborne and Edwards, 2006), with a comprehensive review of the possible interactions by Alexandre *et al*. (2004).

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

Costello *et al*. (2003) proposed a method for testing the compatibility between yeast and bacteria. The aim of the study was to investigate the interaction between these two microorganism populations without the effect of extrinsic grape-derived or processing factors like SO_2 additions, modified pH, sugar concentration and the presence of pesticide residues or nutrients. A chemically defined medium was used to successfully characterise the metabolic interactions between the yeast and bacteria and replacement of the synthetic media with Chardonnay juice produced similar results. This could be an effective tool for screening yeast/LAB combinations in advance to ensure compatibility and lack of antagonistic or inhibitory effects. The winemaker also has control over the vinification practices applied during the winemaking process. These decisions can also affect the interaction between the bacteria and yeast culture. **Table 2.2** shows the effect that different vinification procedures and decisions have on LAB as well as yeast/bacteria interactions.

The second factor is the uptake and release of nutrients by the yeast, which will in turn affect the nutrients available for the LAB. At the start of AF, *O. oeni* is inhibited by *S. cerevisiae* due to the rapid uptake of certain grape metabolites from the must by the yeast. These compounds include sterols, amino acids and vitamins (Larsen *et al*., 2003), which result in a nutrient diminished environment for the bacteria. During AF the amino acids and vitamins that are essential for bacterial proliferation are depleted by yeast metabolism to such an extent that the commencement of bacterial growth is delayed until yeast cells lyse (Nygaard and Prahl, 1997; Alexandre *et al*., 2004; Arnink and Henick-Kling, 2005). Yeast autolysis play a vital role in the release of essential nutrients for LAB proliferation and survival (Alexandre *et al*., 2004). Yeast autolytic activity can release amino acids, peptides, proteins, glucans and mannoproteins and release of these

macromolecules are yeast strain dependant (Alexandre *et al*., 2001; 2004). Mannoproteins seem to be of significant importance, as their release can stimulate bacterial growth by adsorbing medium chain fatty acids and thus detoxifying the wine medium. Mannoproteins can also be enzymatically hydrolysed by bacterial enzyme activity, which will enhance the nutritional content of the wine and in turn stimulate bacterial growth (Guilloux-Benatier and Chassagne, 2003; Alexandre *et al*., 2004). Yeast metabolism has a direct effect on the nitrogen concentration available for LAB consumption. Recently Guilloux-Benatier *et al*. (2006) found that proteolytic activity by yeast can effect the nitrogen composition of wine after AF, which in turn affect the ability of *O. oeni* to grow and complete MLF.

Table 2.2 The influence of different winemaking practices on LAB growth (compiled from Edwards *et al*., 1990 and Alexandre *et al*., 2004).

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

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

Comitini *et al*. (2005) related part of the inhibitory effect of *S. cerevisiae* to the production of extracellular compounds via metabolic activity of the yeast, rather than a competition for nutrients. Therefore, the third factor to consider is the ability of the yeast to produce metabolites that can either have a stimulatory or inhibitory/toxic effect on LAB. There are a number of yeast-derived inhibitory compounds, including ethanol, SO_2, medium chain fatty acids and proteins. The first three are the compounds most commonly studied with regards to LAB growth inhibition (Alexandre *et al*., 2004). Osborne and Edwards (2006) found a peptide produced by *S. cerevisiae* inhibited *O. oeni* and that this inhibition is dependant on the presence of SO_2. This study was performed in synthetic medium and the proposed mechanism was the possible disruption of the cell membrane. Similarly, Comitini *et al*. (2005) also reported a LAB inhibitory compound produced by yeast to be

heat and protease sensitive and therefore also of a proteinaceous nature. In a similar study, Nehme *et al*. (2010) reported the inhibition of an *O. oeni* strain by *S. cerevisiae* that resulted in a decrease in the malic acid consumption by the LAB strain. This inhibition could be attributed, in part, to a peptidic fraction produced by the yeast. **Table 2.3** provides a summary of the major inhibitory compounds produced by yeast.

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

Table 2.3 Yeast activity inhibiting LAB via the production of yeast metabolites.

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

2.5.1.1 Ethanol

Ethanol is the main yeast metabolite formed during AF and due to its adverse effect on LAB growth and metabolic activity, play an integral role in the ability of LAB to survive in the wine environment and accomplish MLF. As with most LAB inhibitory factors, ethanol also demonstrates synergistically inhibiting effects with temperature. The optimal growth temperature of LAB decrease at high ethanol concentrations and elevated temperatures lower the ability of LAB to withstand increased ethanol concentrations (Henick-Kling, 1993; Bauer and Dicks, 2004). Temperatures of 25°C and above, combined with ethanol levels of 10 to 14% $^{v}/_{v}$, almost completely inhibit LAB growth and optimum growth at these ethanol levels occur between 18 and 20°C (Henick-Kling, 1993). Capucho and San Ramao (1994) documented no inhibition of the malolactic activity of *O. oeni* with ethanol levels of up to 12% $^{v}/_{v}$, but saw an inverse correlation between the growth of *O. oeni* and increasing ethanol concentrations (Davis *et al*., 1988; Henick- Kling, 1993; Alexandre *et al*., 2004; Bauer and Dicks, 2004).

It is generally acknowledged that all *O. oeni* strains are able to survive and proliferate in 10% $^{v}/_{v}$ ethanol at pH 4.7 (Britz and Tracey, 1990). G-Alegría *et al*. (2004) reported the ability of *O. oeni* and *Lb. plantarum* strains to grow at 13% $^{v}/_{v}$ ethanol and Henick-Kling (1993) stated that

ethanol concentrations exceeding 14% $^{v}/_{v}$ inhibit the growth of *O. oeni*. The degree to which LAB are able to tolerate ethanol concentrations are strain dependant, as well as being contingent upon the activation steps before inoculation in the wine (Britz and Tracey, 1990).

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

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

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

2.5.1.2 Sulphur Dioxide

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

The mechanism by which SO_2 inhibit LAB include rupturing of disulphide bridges in proteins as well as reacting with cofactors like NAD^+ and FAD, thereby affecting the growth of LAB (Romano and Suzzi, 1993; Carreté *et al.,* 2002). The antimicrobial activity of SO_2 can also influence the

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

There are various compounds, primarily carbonyl compounds, including acetaldehyde, α- ketoglutaric acid and pyruvic acid, that are able to bind SO_2 resulting in the bound form which demonstrates weaker antimicrobial activity (Henick-Kling, 1993). Besides being sensitive to inhibition by the molecular form of SO_2, LAB also possess the ability to liberate SO_2 from acetaldehyde-bounded sulphur, which then prevents further growth of the bacteria and could result in stuck or sluggish MLF (Fornachon, 1963; Osborne *et al.,* 2000; 2006).

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

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

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

ATPase activity by more than 50% and MLF took 40 days to complete in the presence of 5 mg/L free SO_2. For the control and inhibition of LAB, Henick-Kling (1993) suggests maintaining levels of free SO_2 above 10 mg/L and a total SO_2 concentration of above 30 mg/L. Due to the crucial effect that pH has on the form of SO_2 present, García-Ruiz *et al*. (2008) recommend the following concentrations of free SO_2 to inhibit LAB: 10 to 30 mg/L for pH 3.2 to 3.6, 30 to 50 mg/L for pH 3.5 to 3.7 and 100 mg/L for wines with a pH of over 3.7.

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

2.5.1.3 Medium chain fatty acids

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

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

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

Selection of the most suitable yeast strain is imperative to the eventual success of MLF in wine. Care should be taken to choose a yeast strain that is compatible with the strain of LAB, resulting in no or very little antagonistic effect between the yeast/bacteria pairing. This includes a yeast strain that produces very low levels of SO_2 and medium chain fatty acids.

2.5.2 pH

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

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

It is clear that the pH of wine has a number of decisive affects on MLF and LAB. Besides the direct influence of pH, the relationship between pH and SO_2, as previously discussed, is also crucial in understanding the affect of these parameters on the survival of LAB in wine.

2.5.3 TEMPERATURE

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

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

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

2.5.4 PHENOLIC COMPOUNDS

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

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

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

that the disappearance of hydroxycinnamoyltartaric acids resulted in an increase in the free forms during both spontaneous and inoculated MLF.

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

Although the mechanisms by which phenolic compounds inhibit LAB are not entirely clear, there has been some speculation. Possible mechanisms are based on the interactions of phenolic compounds with cellular enzymes (Campos *et al*., 2003; García-Ruiz *et al*., 2008) and the adsorption of phenols to cell walls (Campos *et al*., 2003). Phenolic compounds could lead to a loss in potassium ions, glutamic acid and intracellular RNA, as well as causing a change in the composition of fatty acids (Rozès and Perez, 1998; García-Ruiz *et al*., 2008).

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

The presence of phenolic compounds also has the potential to influence certain quality parameters in wine. Cavin *et al*. (1993) reported the ability of LAB to metabolise hydroxycinnamic acids which result in the formation of volatile phenols with the potential to produce off-flavours (will be discussed in more detail in aroma section). A strain of *O. oeni* studied by Campos *et al*. (2009) was able to produce higher concentrations of acetate in the presence of phenolic acids. This could

be due to enhanced citric acid metabolism at the expense of sugar consumption as documented by Rozès *et al*. (2003). It was also found that this phenomenon is strain dependant. In contrast, Reguant *et al*. (2000) found that gallic acid was able to delay or totally inhibit the formation of acetic acid from citric acid. Tannase activity has also been found in *Lb. plantarum* strains (not in *O. oeni*). Tannase activity allows the hydrolysis of ester bonds in hydrolysable tannins. This reaction releases gallic acid and glucose. Tannase activity could potentially play a role in reducing astringency and haze formation in wine (Vaquero *et al*., 2004).

2.5.5 LYSOZYME

Lysozyme is an enzyme obtained from hen egg white which has been proposed as an alternative to SO_2 for the control of LAB and to delay MLF. This enzyme is highly effective against Gram-positive bacteria (McKenzie and White, 1991; Gerbaux *et al*., 1997; Bartowsky, 2003; Bartowsky *et al*., 2004). The enzyme acts by splitting the *β*-(1-4) linkage between N-acetyl muramic and N-acetyl-glucosamine, components of the peptidoglycan in the bacterial cell wall, leading to lysis and death (McKenzie and White, 1991; Bartowsky *et al*., 2004). Both the susceptibility of LAB, as well as the dosage of lysozyme, are important in determining the efficiency of lysozyme in inhibiting LAB and MLF (Bartowsky, 2003).

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

In a study by Bartowsky *et al*. (2004), lysozyme stability as well as the sensorial impact of lysozyme in bottled wines of Riesling, Cabernet Sauvignon and Shiraz were investigated. A fine red coloured pigment was observed in the Cabernet and Shiraz, likely due to the formation of complexes between lysozyme and coloured phenolic pigments. While no precipitate was observed in the Riesling, the lysozyme did cause heat instability or haze formation in the white wine. There was no detectable lysozyme activity in the red wines after two days, attributed to the complex formation and precipitation, while 76 to 82% residual activity remained in the Riesling after six months. Despite the fact that a colour difference could be observed due to the pigment

precipitation, the sensory panel could not distinguish between the lysozyme treated and untreated wines based on wine aroma and flavour.

The use of lysozyme is an alternative option to SO_2 for the control and inhibition of the indigenous LAB population.

2.6 IMPACT OF MALOLACTIC FERMENTATION ON WINE AROMA

Various studies have shown that MLF has the potential to alter the aroma profile of a wine by the modification or production of flavour-active compounds as depicted in **Figure 2.1** (Davis *et al*., 1985; Nielsen and Richelieu, 1999; Maicas *et al*., 1999; Lonvaud-Funel, 1999; Gámbaro *et al*., 2001; Bartowsky *et al*., 2002b; Bartowsky and Henschke, 2004; D'Incecco *et al*., 2004; Swiegers *et al*., 2005).

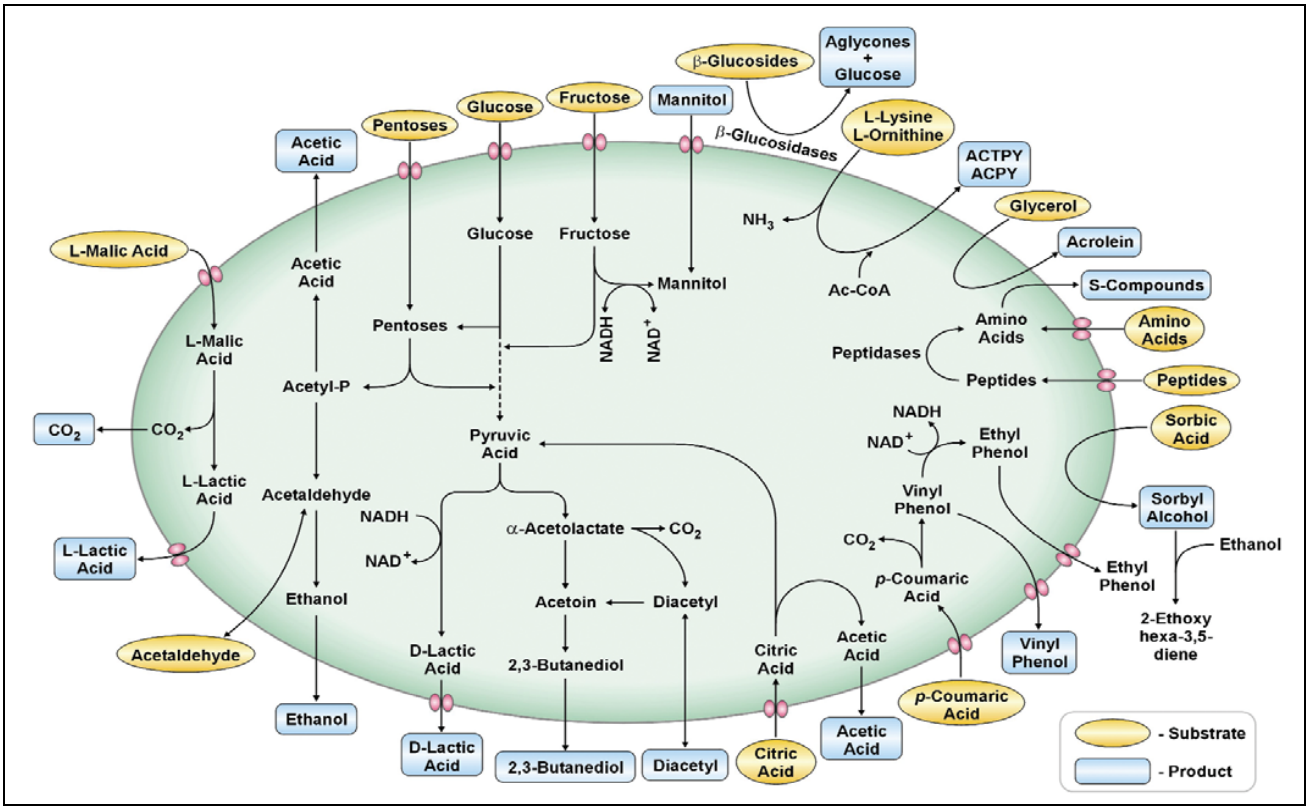

Figure 2.1 A schematic representation of the production and modification of flavour-active compounds by LAB (Swiegers *et al*., 2005).

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

2 to 3 years after bottling of the wine. The extensive bottling ageing period could have resulted in the modification of the wine aroma profile.

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

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

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

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

2.6.1. CARBONYL COMPOUNDS

Diacetyl (2,3-butanedione) is a diketone that contributes buttery, nutty and butterscotch characters to the wine as well as a yeasty character to sparkling wines, during MLF (Bartowsky and Henschke, 1995; Martineau *et al*., 1995; Bartowsky *et al*., 2002b; Bartowsky and Henschke, 2004). It is considered one of the most important aroma compounds produced during MLF (Bartowsky and Henschke, 1995; Lonvaud-Funel, 1999). Diacetyl is formed as an intermediate during the

metabolism of citric acid by the LAB present during MLF (Bartowsky *et al*., 2002b; Bartowsky and Henschke, 2004) (**Figure 2.2**). During carbohydrate metabolism by LAB, pyruvate is reduced to lactate to maintain the redox balance of the bacterial cell. When additional pyruvate is produced as a result of the citric acid metabolism in the absence of sugar, pyruvate is redirected to the production of acetoin and butanediol. Pyruvic acid is reductively decarboxylated to diacetyl via α-acetolactate (Lonvaud-Funel, 1999; Bartowsky *et al*., 2002b; Bartowsky and Henschke, 2004; Costello, 2006). Due to the fact that diacetyl is chemically unstable, it is further reduced to acetoin, which in turn can be reduced to 2,3-butanediol (Bartowsky *et al*., 2002b; Costello, 2006).

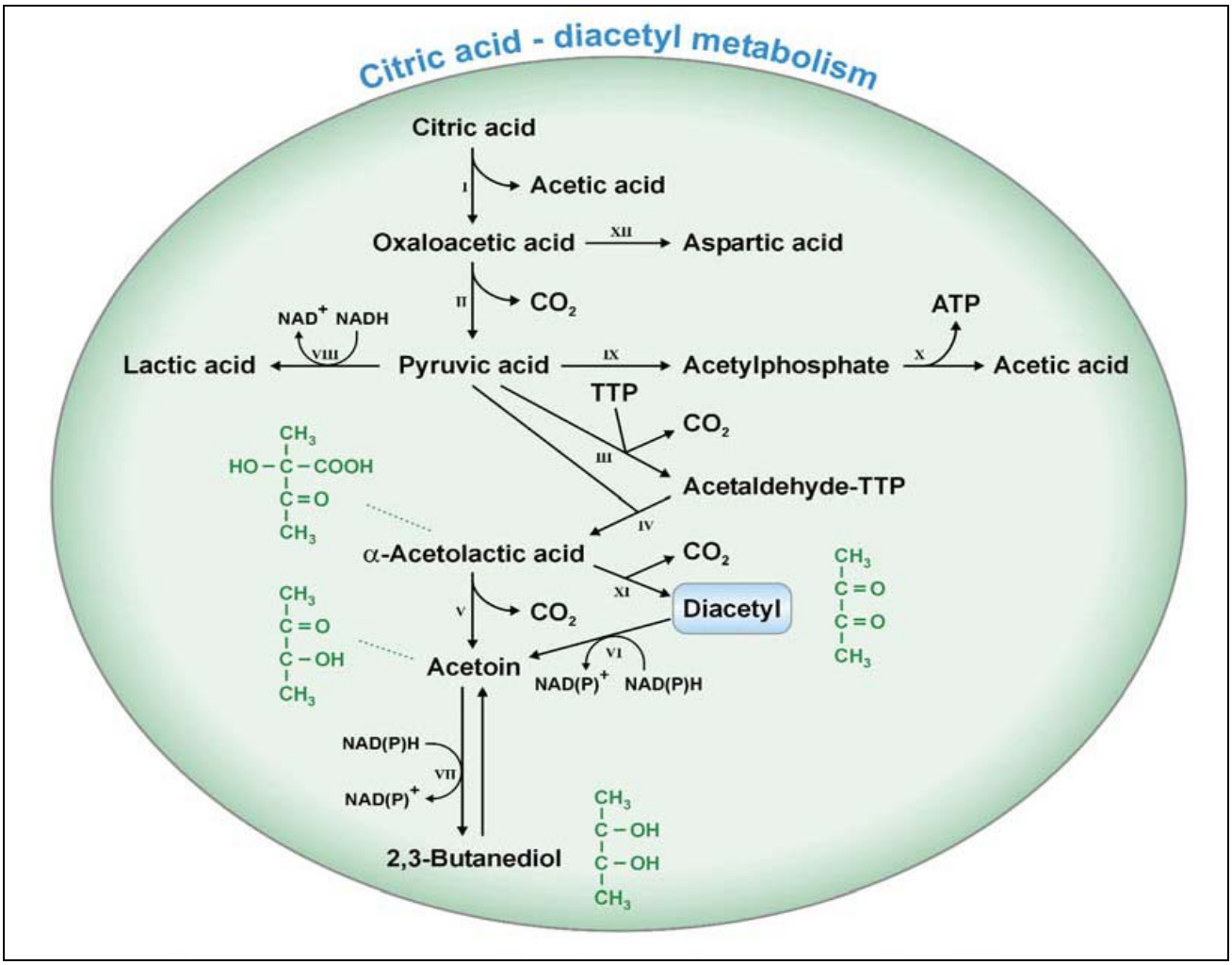

Figure 2.2 A schematic representation of citric acid metabolism and the synthesis of diacetyl in LAB (Swiegers *et al*., 2005).

The accumulation of diacetyl and acetoin is dependant on the rate of MLF. Lower levels of diacetyl and acetoin are produced by a higher MLF rate. Maicas *et al*. (1999) found decreased levels of diacetyl after MLF, but increased levels of 2,3-butanediol due to the enzymatic reduction of diacetyl by LAB. This conversion has a direct effect on wine aroma, due to the fact that acetoin and 2,3-butanediol have higher threshold values, approximately 150 mg/L (Francis and Newton, 2005) and 600 mg/L (Bartowsky and Henschke, 2004), respectively, and are therefore considered

to contribute to the butter aroma to a lesser extent (Bartowsky *et al*., 2002b). In contrast, diacetyl has an odour threshold of approximately 0.2 mg/L in Chardonnay, 0.9 mg/L in Pinot noir and 2.8 mg/L in Cabernet Sauvignon (Martineau *et al*., 1995). Francis and Newton (2005) reported diacetyl levels of 0.2 to 1.84 mg/L generally found in young red wines and 1.25 to 3.39 mg/L in aged red wines. When concentrations exceed 5-7 mg/L, the buttery attribute is overpowering and this character is seen as undesirable, whereas concentrations between 1-4 mg/L can contribute to the buttery and butterscotch aroma and add to the complexity of the wine (Bartowsky and Henschke, 1995, 2004; Swiegers *et al*., 2005). The sensory perception of diacetyl is highly dependant on a number of factors, including the style, age and type of wine (Swiegers *et al*., 2005; Costello, 2006) as well as the presence of other compounds that are able to react with diacetyl e.g. SO_2 (Martineau *et al*., 1995; Bartowsky *et al*., 2002a; Bartowsky and Henschke, 2004; Swiegers *et al*., 2005). **Table 2.4** lists the various ways to manipulate the diacetyl content during the winemaking process.

Table 2.4 Factors which influence the diacetyl content of wine (Martineau and Henick-Kling, 1995; Bartowsky *et al*., 2002b; Bartowsky and Henschke, 2004; Saguir *et al*., 2009).

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

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

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

2.6.2 ESTERS

Esters are important in determining wine aroma and are associated with fruity aromas in wine. The two main groups of fermentation-derived esters that have been associated with wine fruitiness are acetate esters and ethyl fatty acid esters. Ethyl fatty acid esters are formed by the enzymatic esterification of activated fatty acids formed during lipid biosynthesis. Acetate esters are formed through the condensation of higher alcohols with acetyl-CoA (Matthews *et al*., 2004; Ugliano and Henschke, 2008). Even though the esterase activity of LAB are still being evaluated, it is clear that MLF and wine LAB have the ability to alter the ester content (Matthews *et al*., 2004). The extent of this alteration is still unclear, with both increases and decreases in ester concentrations being observed in the literature. MLF is generally associated with increases in the concentration of ethyl esters, including ethyl lactate, ethyl acetate, ethyl hexanoate and ethyl octanoate (De Revel *et al*., 1999; Delaquis *et al*., 2000; Liu, 2002; Swiegers *et al*., 2005; Jeromel *et al*., 2008) as well as diethyl succinate. The modulation of aromatic esters by microbial populations has recently been reviewed by Sumby *et al*. (2009). **Table 2.5** contains some of the esters, other than ethyl lactate and diethyl succinate, associated with MLF and possible aromas that they can contribute to wine.

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

Table 2.5 Concentrations, odour quality and thresholds of some of the other esters found in wine that contribute to the aroma during MLF (compiled from Peinado *et al*., 2004; Francis and Newton, 2005; Vilanova and Martínez, 2007).

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

- not reported above threshold in any study

Maicas *et al*. (1999) found an increase and decrease in the ester concentration according to the choice of bacteria strain. They reported increases in isoamyl acetate, ethyl caproate and 2-phenylethyl acetate. Gambaro *et al*. (2001) found that ethyl- and acetate ester levels decreased during MLF, but these changes were also dependant on the strain of bacteria used. This coincided with a significant decrease in sensory descriptors like 'berry fruit' and 'fresh vegetative'. Jeromel *et al*. (2008) also saw a decrease in isoamyl acetate and 2-phenylethyl acetate due to MLF. Delaquis *et al*. (2000) found an increase in the concentration of ethyl acetate and 3-methyl-1-butyl, which was influenced by the choice of LAB culture. In contrast, ethyl 2-hydroxypropanoate was not influenced by the choice of LAB culture. Ugliano and Moio (2005) studied the effect of four different malolactic starter cultures of *O. oeni* on the concentration of yeast-derived volatile compounds.

MLF increased levels of C_4-C_8 ethyl fatty acid esters and 3-methylbutyl acetate, depending on the bacteria strain used. The total increase in ethyl fatty acid esters were generally larger than the increase observed for the acetate esters.

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

2.6.3 GRAPE-DERIVED COMPOUNDS

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

McMahon *et al*. (1999) found no glycosidase activity in commercial *O. oeni* cultures, whereas Mansfield *et al*. (2002) saw *β*-glucosidase activity in a model system, but none of the strains were active on Viognier grape glycosides. This could imply that the cultivar has an influence on the enzyme activity. In contrast, Grimaldi *et al*. (2000), Ugliano *et al*. (2003) as well as Ugliano and Moio (2006) found a decrease in the concentration of total glycosides and an increase in the free compounds after MLF with *O. oeni*. Boido *et al*. (2000) found that due to the *β*-glucosidase activity of *O. oeni*, the free aroma compounds released from their glycosylated forms increased. They postulated that the increase was smaller than expected due to stable associations between released aroma compounds and bacterial polysaccharides. This could be a possible cause as to why D'Incecco *et al*. (2004) observed limited liberation of aroma compounds in Chardonnay

glycosidic extract during MLF. The degree to which this enzymatic hydrolysis takes place is dependant on the bacterial strain, chemical structure of the substrates and growth phase of the bacteria. Glycosidase activity is also influenced by pH, temperature, sugars and ethanol (Grimaldi *et al*., 2000; 2005b). The acidic conditions found in wine may denature or inhibit the enzymatic activity. However, *O. oeni* retained up to 80% of *β*-glucosidase activity at pH 3.5 (Grimaldi *et al*., 2000). Barbagallo *et al*. (2004) also showed the ability of wild *O. oeni* strains to retain their *β*-glucosidase activity under wine conditions. Mtshali (2007) screened and characterised the *β*-glucosidase enzyme in LAB isolated from South African wines. The enzyme specific primers amplified the gene with a size corresponding to 1392 bp, with 40% of the isolates testing positive for the presence of the gene, none of which were *O. oeni* strains. This supports the investigation of alternative LAB genera for possible use in a starter culture that could assist in the liberation of grape-derived aroma compounds.

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

2.6.4 VOLATILE SULPHUR COMPOUNDS

Sulphur containing compounds associated with MLF as a result of LAB metabolism, have not been investigated until as recently as 2004. Pripis-Nicolau *et al.* (2004) were the first to demonstrate the ability of wine LAB to metabolise methionine to produce volatile sulphur compounds during MLF. The formation of volatile sulphur compounds in fermented foods has recently been reviewed by Landaud *et al*. (2008), including volatile sulphur compounds associated with wine. The precise mechanism and biochemical pathways that make up sulphur metabolism in wine LAB have not been fully investigated and little is known. **Figure 2.3** displays the sulphur metabolism in LAB and it is assumed that wine LAB will share some of the characteristics and pathways characterised in other LAB, specifically LAB from the dairy industry (Liu *et al*., 2008).

Vallet *et al*. (2008) proposed the possible pathway by which these compounds are formed by *O. oeni*. The metabolism of methionine by LAB leads to the formation of methanethiol, dimethyl disulphide, 3-(methylsulphanyl)propan-1-ol (also known as methionol) and 3-(methylsulphanyl)propionic acid. The formation of these compounds is important in the complexity of wine aroma because of their characteristic and powerful odours shown in **Table 2.6**. Increasing concentrations of these sulphur compounds will impart negative aromas to the wine, but concentrations below or close to threshold will add to complexity. The threshold values of some of the most important sulphur compounds are listed in **Table 2.6**. Concentrations of methanethiol and 3-(methylsulphanyl)propan-1-ol above their thresholds are usually associated with reduction off-flavours (Pripis-Nicolau *et al*., 2004). The production of these volatile sulphur compounds are also strain dependant as well as genus dependant, with *O. oeni* having a higher capacity for producing these compounds, compared to the species of *Lactobacillus* (Pripis-Nicolau *et al*., 2004).

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

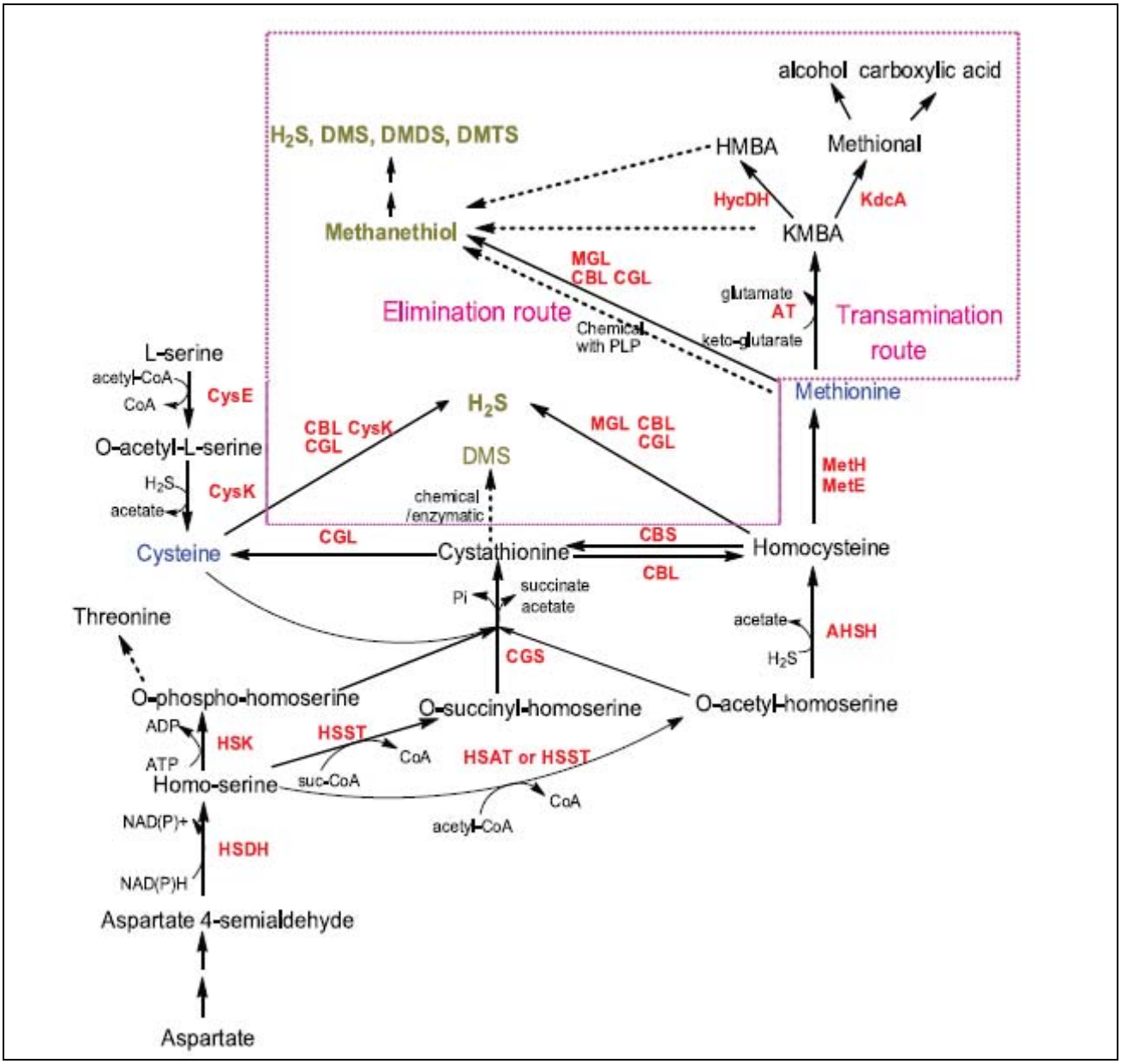

Figure 2.3 Cysteine and methionine metabolism in LAB (Liu *et al*., 2008). Both the biosynthesis and degradation pathways of cysteine and methionine are included in this map, while the catabolic reactions are framed. Key sulphur-containing flavour compounds are coloured green.

Table 2.6 Examples of some volatile sulphur compounds found in wine and produced by LAB during MLF (Landaud *et al*., 2008).

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

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

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

2.6.5 NITROGEN CONTAINING COMPOUNDS

Wine LAB are able to produce heterocyclic volatile nitrogen bases responsible for the 'mousy' off-flavour encountered in spoiled wines. Three main compounds are involved: 2-acetyl-1-pyrroline (ACPY), 2-ethyltetrahydropyridine (ETPY) and 2-acetyltetrahydropyridine (ACTPY) (Lonvaud-Funel, 1999; Costello *et al*., 2001). The formation of these compounds is mostly associated with heterofermentative LAB, such as *O. oeni*, some *Lactobacillus* species and *Leuc. mesenteroides*, through the metabolism of certain amino acids, especially ornithine and lysine (Costello *et al*., 2001; Swiegers *et al*., 2005). ACTPY and ACPY are the most powerful odorants compared to ETPY, with thresholds in water of 1.6 µg/L and 0.1 µg/L, respectively. Wines that are considered spoiled by the ‘mousy’ off-flavour, generally contain amounts of 2.7 to 18.7 µg/L of ATPY, up to 7.8 µg/L ACPY and 4.8 to 106 µg/L of ACTPY. These compounds are

either present in combination or individually. The availability of the precursors lysine and ornithine has a significant impact on the ability of LAB to produce these compounds (Costello and Henschke, 2002). Costello and Henschke (2002) also found that the lack of ethanol drastically reduces the ability of *Lb. hilgardii* to produce N-heterocycles, whereas elevated concentrations of acetaldehyde stimulates formation. LAB differ in their preference for the formation of the different nitrogen heterocyclic compounds. *Oenococcus oeni* favours the production of the least flavour active ETPY, the heterofermentative lactobacilli the formation of ACTPY and the homofermentative pediococci the formation of the most flavour active ACPY. In general, the heterofermentative LAB show the highest ability to produce nitrogen-heterocycles and mousy off-flavour (Swiegers *et al*., 2005).

2.6.6 VOLATILE PHENOLS

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

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

2.6.7 ACETIC ACID

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

There are two proposed mechanisms by which LAB can increase acetic acid levels in wine. If MLF commence before the completion of AF, the LAB are able to ferment hexoses that have not been completely fermented by the yeast. *Oenococcus oeni* is a heterofermentative strain and will not only produce ethanol and CO_2, but also acetic acid and D-lactic acid, via the 6-PG/PK pathway. As a consequence, the VA increase (Lonvaud-Funel, 1999; Swiegers *et al*., 2005). During the formation of diacetyl, LAB can also produce acetic acid during the first reaction of the citric acid metabolic pathway catalysed by the citrate lyase enzyme (Bartowsky *et al*., 2002b; Bartowsky and Henschke, 2004). The rate of acetic acid accumulation is dependant on the rate of MLF, with higher concentrations of acetic acid formed in conjunction with a higher MLF rate (Lonvaud-Funel, 1999).

2.6.8 VOLATILE FATTY ACIDS

Volatile fatty acids are formed by the hydrolysis of tri-, di- and monoacylglycerols (lipids) (Liu, 2002). Wine consists of a mixture of straight chain fatty acids and branched chain fatty acids. The straight chain fatty acids are usually referred to as short chain (C_2-C_4), medium chain (C_6-C_{10}) or long chain (C_{12}-C_{18}) fatty acids (Ugliano and Henschke, 2008). As the chain length of fatty acids increase, the volatility decreases and the odour changes from sour to rancid and cheese (Francis and Newton, 2005; Ugliano and Henschke, 2008). **Table 2.7** contain the most common volatile fatty acids found in wine and their possible contribution to the sensory profile of wine.

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

Table 2.7 Concentrations, odour quality and thresholds of volatile fatty acids found in wine (Francis and Newton, 2005).

Acids	Odour quality	Concentration (µg/L) in		Odour Threshold (µg/L)
		Young red wine	Aged red wine	
Isobutyric acid	Rancid, butter, cheese	434 - 2345	3510 - 7682	2300
Isovaleric acid	Sweat, acid, rancid	305 - 1151	1062 - 3507	33.4
Butyric acid	Rancid, cheese, sweat	434 - 4719	2020 - 4481	173
Propionic acid	Pungent, rancid, sweat	-	4160 - 11907	8100
Hexanoic acid	Sweat	853 - 3782	1441 - 5838	420
Octanoic acid	Sweat, cheese	562 - 4667	1095 - 4970	500
Decanoic acid	Rancid, fat	62.1 - 857	290 - 2000	1000

- not reported above threshold in any study

2.6.9 HIGHER ALCOHOLS

Higher alcohols are formed by the decarboxylation and subsequent reduction of *α*-keto acids. The keto acids are produced as intermediates during amino acid biosynthesis and catabolism, the latter referred to as the Ehrlich pathway. Amino acid biosynthesis is responsible for most of the higher alcohols formed during fermentation (Ugliano and Henschke, 2008). At lower concentrations (less than 300 mg/L), higher alcohols can contribute to the complexity and fruity aromas in wine, whereas higher concentrations (above 400 mg/L) could be detrimental to wine aroma and quality due to the harsh chemical-like aromas (Swiegers *et al*., 2005) (**Table 2.8**).

Table 2.8 Concentrations, odour quality and thresholds of some higher alcohols found in wine (Francis and Newton, 2005).

Higher alcohol	Odour quality	Concentration (mg/L) in		Odour Threshold (mg/L)
		Young red wine	Aged red wine	
Isobutanol	Wine, solvent, bitter	25.7 - 86.9	57.2 - 230	40
Isoamyl alcohol	Whiskey, malt, burnt	83.95 - 333	165 - 472	30
2-phenylethyl alcohol	Honey, spice, rose, lilac	9 - 153	24 - 166.6	10-14

Jeromel *et al*. (2008) found that MLF had an insignificant effect on the higher alcohol concentration of wine, except for significant increases in isobutanol and 2-phenylethanol. In contrast, Herjavec *et al*. (2001) found no change in levels of 1-propanol, isobutanol, isoamyl alcohol or 2-phenylethanol. This is supported by Maicas *et al*. (1999) who found the production of isobutanol, 1-propanol, 1-butanol and isoamyl alcohol to be dependant on the strain used to perform MLF. Pozo-Bayón *et al*. (2005) saw increased levels of higher alcohols after MLF, but

none of the increases were significant. The fact that LAB seem to have limited ability to produce fusel alcohols could be beneficial, as most of these compounds impart harsh solvent-like aromas in the wine. The concentration of higher alcohols that have either a positive or negative influence on the wine aroma, is likely to depend on both the aroma intensity of the respective alcohols as well as the style of wine (Ugliano and Henschke, 2008).

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

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

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

2.7 IMPACT OF MALOLACTIC FERMENTATION ON WINE WHOLESOMENESS

2.7.1 BIOGENIC AMINES

Biogenic amines are a group of organic nitrogen-containing compounds. The main biogenic amines associated with wine are putrescine, histamine, tyramine and cadaverine, followed by phenylethylamine, spermidine, spermine, agmatine and tryptamine (Ten Brink *et al*., 1996; Lonvaud-Funel, 2001). The role of biogenic amines in wine and the microorganisms involved in their synthesis, were recently reviewed by Smit *et al*. (2008).

Biogenic amines are formed by certain LAB via the substrate-specific enzymatic decarboxylation of naturally occurring amino acids (Ten Brink *et al*., 1996; Lonvaud-Funel, 2001).

These compounds are of importance in wine due to their potential toxicological effects in sensitive humans. These include symptoms like headaches, hypo- or hypertension, cardiac palpitations and in extreme cases even anaphylactic shock (Shalaby, 1996). The presence of alcohol, SO_2 and other amines could potentially amplify the toxic effect of certain biogenic amines (Fernandes and Ferreira, 2000; Volschenk *et al*., 2006). There are various factors that influence the biogenic amine content. These factors include the amino acid composition, the microflora present in the wine and the ability of the microflora to decarboxylate amino acids. All parameters that favour bacterial growth will favour biogenic amine formation (Lonvaud-Funel and Joyeux, 1994; Volschenk *et al*., 2006).

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

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

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

wine. Rosi *et al*. (2009) studied 26 strains of *O. oeni* for their biogenic amine formation ability in synthetic medium and wine. These authors found that the concentration of histamine and tyramine formed by *O. oeni* were dependant on the bacterial strain, the effect of the yeast strain on the wine composition, the length of bacteria-yeast contact time after MLF completion, as well as the screening method used for biogenic amine determination.

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

The ability to produce biogenic amines is used as a screening criterion in the selection of LAB starter cultures. It is imperative to be able to identify strains with the potential to produce biogenic amines. The ingestion of biogenic amines, histamine in particular, can lead to various health reactions in sensitive humans. These include headaches, low blood pressure, diarrhoea and even heart palpitations. Phenylethylamine and tyramine can cause symptoms of high blood pressure and migraines. Putrescine and cadaverine, besides being able to enhance the toxicity of histamine, tyramine and phenylethylamine, can also have a detrimental effect on wine quality by imparting flavours of putrefaction and rotten meat, respectively (Shalaby, 1996; Palacois, 2006). Le Jeune *et al*. (1995) developed a detection system for histamine producing LAB strains and more recently, Marcobal *et al*. (2005) selected three primer pairs to use in a multiplex polymerase chain reaction (PCR) assay to simultaneously detect histamine, tyramine and putrescine producing LAB. The assay yielded a 367 bp DNA fragment from histidine decarboxylases (*hdc*) (primer pair JV16HC/JV17HC), a 924 bp fragment from tyrosine decarboxylases (*tdc*) (primer pair P1-rev/P2-for) and a 1446 bp fragment from ornithine decarboxylases (*odc*) (primer pair 3/16). The

first PCR detection for cadaverine producing LAB has also been developed (De las Rivas *et al*., 2006).

In a study of the potential of commercial cultures to produce tyramine, histamine and putrescine, it was found that none of the commercial starter cultures produced biogenic amines (Moreno-Arribas *et al*., 2003). In a study comparing spontaneous and inoculated MLF in Spanish red wine, the incidence of biogenic amines was reduced in the inoculated MLF (Martín-Álvarez *et al*., 2006). Similarly, Izquierdo Cañas *et al*. (2007) determined that histamine, tyramine and putrescine concentrations increased from 106% to 174% in Spanish wines due to spontaneous MLF.

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

2.7.2 ETHYL CARBAMATE

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

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

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

2.8 MALOLACTIC FERMENTATION MONITORING

2.8.1 MONITORING OF MALIC ACID CONCENTRATION

The decrease in malic acid or increase in lactic acid is mostly used to monitor the progression of MLF. Analytical techniques are useful for monitoring the malic acid concentration. A summary of the monitoring techniques as well as the advantages and disadvantages are provided in **Table 2.9**. There are various techniques available for the monitoring of the changes in the malic acid concentration during MLF. These methods include chromatography, reflectance and enzymatic assays, as well as analytical techniques like Fourier-transform infrared (FT-IR) spectroscopy and Capillary electrophoresis (CE) or the use of High-performance liquid chromatography (HPLC). These techniques differ in their accuracy, time needed for analysis as well as the cost involved.

Chromatography, like paper chromatography (PC) and thin layer chromatography (TLC), is the method most often implemented in wineries due to the low cost involved. Unfortunately, these methods are not as accurate as some of the analytical techniques. The more accurate methods usually involve the acquirement of expensive equipment like a CE and HPLC. In order to accurately monitor the progression of MLF, fast and accurate results are required. The use of an enzymatic kit could address both of these aspects. Although the cost involved is still relatively high, it is still less expensive than acquiring machinery like an HPLC or a FT-IR spectrometer. The commercial scale of the cellar and the amount of samples to be analysed on a regular basis will greatly influence the selection of the most suitable malic acid monitoring technique.

Table 2.9 A summary of the most popular methods for malic acid and MLF monitoring including the advantages and disadvantages of each method [compiled from Theodore (2006) and Kollar and Brown (2006)].

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

2.8.2 MONITORING OF MICROBIAL POPULATION

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

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

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

There are various molecular techniques available that aid in the characterisation of LAB and add to the knowledge of these bacteria and their role in the winemaking process (Lonvaud-Funel, 1995). These techniques enable us to identify microbes, differentiate LAB from each other as well as distinguish between different strains within the same species (**Table 2.10**) (Bartowsky *et al*., 2003). Some of these techniques include: DNA-DNA hybridisation, 16S and 23S rRNA sequence analysis, DNA-fingerprinting and pulse-field gel electrophoresis (PFGE) as well as PCR-based DNA fingerprinting known as randomly amplified polymorphic DNA (RAPD) analysis (Bartowsky *et al*., 2003). These techniques are used to identify and differentiate between LAB (Zapparoli *et al*., 1998; Bartowsky and Henschke, 1999).

Table 2.10 A summary of molecular techniques available for monitoring and characterisation of the microbial population during MLF.

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

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

2.9 CONCLUSIONS

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

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

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

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

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

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

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Chapter 3

Research results

Selection and characterisation of lactic acid bacteria for possible use as a malolactic fermentation starter culture

This manuscript will be submitted for publication in
International Journal of Food Microbiology

3. RESEARCH RESULTS

Selection and characterisation of lactic acid bacteria for possible use as a malolactic fermentation starter culture

3.1 INTRODUCTION

Malolactic fermentation (MLF) in wine is a deacidification process via the decarboxylation of malic acid by the malolactic enzyme and is a result of the metabolic activity of lactic acid bacteria (LAB). LAB usually present in wine include species from the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Wibowo *et al.,* 1985). *Oenococcus oeni* is the species of LAB that is most commonly responsible for MLF in wine and have shown to be able to most successfully survive the challenging wine environment. Some of these challenges include high alcohol concentrations, low pH, low temperatures and the presence of sulphur dioxide (Wibowo *et al*., 1985; Davis *et al.,* 1988; Drici-Cachon *et al.,* 1996; Lonvaud-Funel, 1999). Currently, *O. oeni* is the only LAB used in commercial starter cultures for MLF. Some *Lactobacillus* species, including *Lactobacillus plantarum*, have exhibited the ability to survive the harsh wine conditions (Drici-Cachon *et al.,* 1996; Lonvaud-Funel, 1999; G-Alegría *et al.,* 2004; Pozo-Bayón *et al.,* 2005). *Lactobacillus plantarum* is the species that has shown the most promise for use in a starter culture. It has been previously considered for use in a MLF starter culture (Viniflora, Chr. Hansen, Hørsholm, Denmark) and is one of the dominant *Lactobacillus* species found in grape must (Du Plessis *et al*., 2004). This species of LAB also shows a more diverse enzymatic profile than *O. oeni* (Mtshali, 2007; Matthews *et al*., 2004; Spano *et al*., 2005), which could play an important role in the modification of the wine aroma profile (Guerzoni *et al*., 1995; Pozo-Bayón *et al.,* 2005; Swiegers *et al.,* 2005; Matthews *et al.,* 2006).

Winemakers are starting to understand the value of inoculating for MLF using a commercial starter culture. By using these cultures, it is possible to increase the chances of successful initiation and completion of fermentation whilst reducing the risks associated with spontaneous MLF (Davis *et al*., 1985; Fugelsang and Zoecklein, 1993; Henick-Kling, 1995). The use of these cultures can ensure a positive flavour contribution during MLF and reduce the risk of spoilage bacteria that are often associated with the production of undesirable or off-flavours and have a detrimental effect on the quality of the wine (Bartowsky and Henschke, 1995; Fugelsang and Edwards, 1997).

These above mentioned attributes are necessary traits in a commercial starter culture and therefore provide essential selection and screening criteria for LAB strains that are being considered for use as a starter culture. Some of these criteria include the inability to produce biogenic amines, the lack of off-flavour or off-odour production, the ability to tolerate low pH, high ethanol and sulphur dioxide (SO_2) concentrations and good growth characteristics under

winemaking conditions (Wibowo *et al*., 1985; Kunkee, 1991; Fugelsang and Zoecklein, 1993; Henick-Kling, 1993; Le Jeune *et al*., 1995; Drici-Cachon *et al*., 1996; Lonvaud-Funel, 2001; Marcobal *et al*., 2004; Volschenk *et al*., 2006). The selection and screening of LAB strains under wine conditions are also of fundamental importance, due to the fact that LAB strains demonstrate significant differences in their fermentation capabilities and growth characteristics (Britz and Tracey, 1990; Henick-Kling, 1993).

The inability to produce biogenic amines is one of the essential criteria when isolates are being considered for use in a starter culture. The role of LAB in the production of biogenic amines during MLF has been established (Lonvaud-Funel and Joyeux, 1994; Moreno-Arribas *et al*., 2000; Liu, 2002; Marcobal *et al*., 2006; Volschenk *et al*., 2006; Landete *et al*., 2007). LAB are able to synthesise biogenic amines via the substrate-specific enzymatic decarboxylation of amino acids that are naturally present in wine (Ten Brink *et al*., 1990; Lonvaud-Funel, 2001). This is an important consideration due to the potential toxic effects that are associated with biogenic amine ingestion. In sensitive humans, these compounds can cause symptoms of headaches, hypo- or hypertension, cardiac palpitations and in extreme cases even anaphylactic shock (Shalaby, 1996). Another important consideration is the negative aroma descriptors associated with some of the biogenic amines. Putrescine, the most abundant biogenic amine in wine, can reduce sensorial quality at 15 to 20 mg/L and 20 to 30 mg/L in white and red wines, respectively (Arena & Manca de Nadra, 2001). Spoilage LAB, most commonly species of *Pediococcus* and *Lactobacillus,* are generally responsible for the formation of biogenic amines (Moreno-Arribas and Polo, 2008). Martín-Álvarez *et al*. (2006) compared spontaneous and inoculated MLF in Spanish red wines and found lower concentrations of biogenic amines in wines that were inoculated for MLF. In another study, spontaneous MLF led to a 68% increase in histamine, tyramine and putrescine concentrations (Izquierdo Cañas *et al*., 2007). The increase in biogenic amines due to spontaneous MLF is an important consideration in the decision to inoculate for MLF with a starter culture.

A further consideration is the influence that the LAB have on the aroma profile of wine undergoing MLF. It has been established that LAB alter the aroma profile of wine via the modification and production of flavour-active compounds (Davis *et al*., 1985; Lonvaud-Funel, 1999; Maicas *et al*., 1999; Nielsen and Richelieu, 1999; Gámbaro *et al*., 2001; Bartowsky *et al*., 2002; Bartowsky and Henschke, 2004; D'Incecco *et al*., 2004; Swiegers *et al*., 2005). There are various enzymes originating from LAB that could potentially contribute to the sensory profile of wine undergoing MLF. Some of these enzymes include *β*-glucosidase, phenolic acid decarboxylase (PAD), citrate lyase, esterase and protease (Mtshali, 2007).

Various potentially volatile aroma compounds which are grape derived exist, but are bound to a sugar molecule; rendering these compounds sensorially inactive (D'Incecco *et al*., 2004; Bartowsky *et al*., 2004; Swiegers *et al*., 2005). The sugar moiety bound to these compounds usually constitutes a glucose molecule, which means that LAB with the ability to demonstrate

β-glucosidase activity could release these volatile compounds to become odour-active, which enable them to contribute to the sensorial profile of the wine (Grimaldi *et al*., 2000; Boido *et al*., 2002; Liu, 2002; D'Incecco *et al*., 2004; Barbagallo *et al*., 2004; Matthews *et al*., 2004). The glycosidic activity is influenced by pH, temperature, sugars and ethanol and may be denatured or inhibited by the acidic conditions found in wine (Grimaldi *et al*., 2000; 2005). However, Grimaldi *et al*. (2000) demonstrated the ability of *O. oeni* to retain up to 80% of *β*-glucosidase activity at pH 3.5 and Barbagallo *et al*. (2004) also found wild *O. oeni* strains able to retain *β*-glucosidase activity under wine conditions. Isolates in malolactic starter cultures that are able to liberate sugar-bound aroma compounds could potentially contribute to the overall aroma profile of the wine.

There are various phenolic compounds present in wine, specifically the phenolic acids *p*-coumaric acid and ferulic acid. LAB are able to metabolise these substrates, via hydroxycinnamic- or phenolic acid decarboxylases, to their vinyl derivatives. These vinyl derivatives can be reduced to produce the volatile phenols 4-ethylphenol and 4-ethylguaiacol. These compounds impart negative sensorial qualities to wine, including animal and medicinal aromas, horse sweat, horse stable, barnyard and elastoplast aromas (Cavin *et al*., 1993; Lonvaud-Funel, 1999; Swiegers *et al*., 2005). Even if LAB strains possess the potential to produce volatile phenols, it is not clear if they are able to produce sensorially significant levels of these compounds (Gámbaro *et al*. 2001; Swiegers *et al*., 2005).

Diacetyl (2,3-butanedione) is considered to be one of the most important aroma compounds produced by LAB during MLF (Bartowsky and Henschke, 1995; Lonvaud-Funel, 1999) and is responsible for the buttery, butterscotch and nutty descriptors usually associated with wines that have undergone MLF (Bartowsky and Henschke, 1995; 2004; Martineau *et al*., 1995; Bartowsky *et al*., 2002). Diacetyl is formed as an intermediate of the citric acid metabolism pathway of LAB and the first reaction in this pathway is catalysed by the citrate lyase enzyme (Bartowsky *et al*., 2002; Bartowsky and Henschke, 2004). Depending on the type and style of wine desired, LAB isolates with the potential to produce diacetyl could be beneficial in adding complexity to the sensorial profiles of wines during MLF.

Esters are very important compounds that contribute to wine aroma and impart fruity aromas, like diethyl succinate, but can also contribute to mouthfeel and the palate of the wine via the presence of esters like ethyl lactate (Maicas *et al*., 1999; Herjavec *et al*., 2001; Ugliano and Moio, 2005). Esterase that originate from wine LAB are responsible for both the biosynthesis and hydrolysis of esters (Matthews *et al*., 2004). *Oenococcus oeni*, as well as species of *Lactobacillus* and *Pediococcus*, are able to hydrolyse esters and Matthews *et al*. (2006) found that significant esterase activity levels remained under wine-like conditions. This implies that esterase that originate from LAB could potentially contribute to the wine aroma profile.

Protease activity in wine LAB have not been fully investigated or characterised. There is a possibility that wine LAB protease activity can degrade proteins found in wine and the metabolism of these proteins could produce amino acids and peptides that could potentially alter the wine

aroma profile. Even though results by Davis *et al*. (1988) found no wine LAB strains that were positive for protease, the concentrations of some amino acids increase during MLF (Wibowo *et al*., 1985; Pozo-Bayón *et al*., 2005), which could imply protease activity by wine LAB (Mtshali *et al*., 2009).

Based on the importance of these aroma compounds and the enzymes responsible for their production, it is important to investigate and evaluate the potential effect that bacterial isolates, which are being considered for use as a malolactic starter culture, could have on the aroma profile and quality of the wine.

The overall aim of the study was to evaluate *O. oeni* and *Lb. plantarum* South African wine isolates for use as MLF starter cultures. The first objective was to assess the malic acid degradation rate in a synthetic wine medium. The second objective was to screen the isolates for biogenic amine producing genes. The third objective was to screen the selected isolates with polymerase chain reaction (PCR) for the presence of wine hydrolysing enzymes such as *β*-glucosidase, PAD, citrate lyase, esterase and protease.

3.2 MATERIALS AND METHODS

3.2.1 BACTERIAL ISOLATES, MEDIA AND CULTURE CONDITIONS

LAB strains selected for use in this study were obtained from two sources. Bacterial isolates, which had been previously identified, were selected from the Institute for Wine Biotechnology (IWBT) culture collection. Bacterial strains were also isolated from spontaneous MLF in Pinotage wine from the Paarl region. **Table 3.1** contains all the bacterial strains used in the initial stage of the project.

Table 3.1 The list of strains that were selected from the IWBT culture collection and isolated from spontaneous MLF. These strains were identified with colony PCR and species-specific primer sets.

Isolate no.	Species name	Isolate no.	Species name
B69	*O. oeni*	2.1	*Lb. plantarum*
D59	*O. oeni*	14	*Lb. plantarum*
D60	*O. oeni*	14.1	*Lb. plantarum*
E53	*O. oeni*	56	*Lb. plantarum*
J65	*O. oeni*	65	*Lb. plantarum*
M69	*O. oeni*	66.1	*Lb. plantarum*
N73	*O. oeni*	68	*Lb. plantarum*
W56	*O. oeni*	69	*Lb. plantarum*
W75	*O. oeni*	70	*Lb. plantarum*
W77	*O. oeni*	71	*Lb. plantarum*
A1 *	*O. oeni*	71.1	*Lb. plantarum*
A2 *	*O. oeni*	73.1	*Lb. plantarum*
B1 *	*O. oeni*	75	*Lb. plantarum*
C1 *	*O. oeni*	76.2	*Lb. plantarum*
S1 *	*O. oeni*	77.1	*Lb. plantarum*
S2 *	*O. oeni*	78.1	*Lb. plantarum*
S3 *	*O. oeni*	80.2	*Lb. plantarum*
S4 *	*O. oeni*	107	*Lb. plantarum*
S5 *	*O. oeni*	109	*Lb. plantarum*
S6 *	*O. oeni*		
S7 *	*O. oeni*		
S8 *	*O. oeni*		
S9 *	*O. oeni*		

* Strains isolated from spontaneous MLF

Lactobacillus plantarum strains were cultivated on De Man, Rogosa and Sharpe (MRS) plates containing 50 g/L MRS broth (Biolab, Merck) and 15 g/L Bacteriological agar (Biolab, Merck). *Oenococcus oeni* strains were cultivated on MRS plates containing 50 g/L MRS and 20 g/L Bacteriological agar supplemented with 10% preservative free tomato juice (All Gold, South Africa) (MRST) with pH adjusted to 5.0 with hydrochloric acid (HCl). All plates contained 50 mg/L Delvocid Instant (DSM Food Specialties, The Netherlands) to prevent the growth of yeasts and 25 mg/L Kanamycin sulphate (Roche Diagnostics GmbH, Mannheim, Germany) to suppress the growth of acetic acid bacteria. All LAB were anaerobically cultivated by using Microbiology Anaerocult sheets

in anaerobic jars (Merck, Darmstadt, Germany). Agar plates for the enumeration of *Lb. plantarum* and *O. oeni* strains were incubated at 30°C for 4 and 7 days, respectively.

To evaluate the strains in the synthetic wine medium, *Lb. plantarum* strains were grown at 30°C in MRS broth for 2 days. *Oenococcus oeni* strains were grown at 30°C for four days in filter-sterilised MRS broth containing 50 g/L MRS supplemented with 20% preservative free apple juice (Ceres, South Africa) (MRSA) and with pH adjusted to 5.2 with HCl. For DNA preparation purposes, the LAB strains were grown at 30°C in 1.5% (w/v) glycine (Saarchem, Merck) supplemented broth for 24 hours.

Strains were maintained as culture stocks in 40% (v/v) glycerol (Saarchem, Merck) at -80°C.

3.2.2 IDENTIFICATION OF LACTIC ACID BACTERIA ISOLATES

Identification of the LAB strains, as either *Lb. plantarum* or *O. oeni,* were done using colony PCR with species-specific primers. The identity of 19 of the 24 previously identified *Lb. plantarum* strains from the IWBT culture collection were confirmed using species-specific primers designed by Torriani *et al*. (2001). The 50 µL PCR reaction mixture contained 1x Reaction buffer (Supertherm, Southern Cross Biotechnology), 200 µM dNTP's (Takara, Seperations), 0.5 µM of each primer in the primer set planF/pREV (Whitehead Scientific, South Africa) (**Table 3.2**)**,** 1.5 mM $MgCl_2$ (Supertherm, Southern Cross Biotechnology), 1.25 U DNA polymerase (Supertherm, Southern Cross Biotechnology) and a single bacterial colony.

The identity of 10 of the 17 previously identified *O. oeni* strains from the IWBT culture collection were confirmed with colony PCR using species-specific primers according to the method described by Zapparoli *et al*. (1998). This method was also used for identifying13 of the 15 strains isolated from spontaneous MLF as *O. oeni*. The 50 µL PCR reaction mixture contained 1x Reaction buffer, 200 µM dNTP's, 0.5 µM of each primer in the primer set On1/On2 (**Table 3.2**), 2 mM $MgCl_2$, 1.25 U DNA Polymerase and a single bacterial colony. The identified strains were selected for further use in this study.

Reference strains that had previously been identified with colony PCR served as positive controls in the PCR reaction as well as a negative control which contained no bacterial DNA template. PCR reaction conditions were as described in **Table 3.3**. All PCR reactions were done using a T3 Thermocycler (Whatman Biometra GmbH, Germany). PCR products were analysed by gel electrophoresis in 2% (w/v) agarose (Whitehead Scientific) gels containing ethidium bromide (Sigma-Aldrich). Gels were run at 70V in a 1x TAE buffer (100 mM Tris-Cl, 1 mM EDTA, pH 8.0, 0.20 mM acetate) for approximately 45 minutes. UV transillumination was used for visualising DNA fragments and an Alpha Imager (Alpha Innotech Corporation, San Leandro, California) used for documenting the image. DNA Molecular Weight Marker XIV (Roche) was used as the standard molecular weight marker.

Table 3.2 The species-specific primer sequences for the identification of wine LAB using colony PCR.

Primer name	Primer sequence (5'-3')	Identification	Reference
planF (fwd)	CCGTTTATGCGGAACACCTAA	*Lb. plantarum*	Torriani *et al*. (2001)
pREV (rev)	TCGGGATTACCAAACATCAC		Torriani *et al*. (2001)
On1 (fwd)	TAATGTGGTTCTTGAGGAGAAAAT	*O. oeni*	Zapparoli *et al*. (1998)
On2 (rev)	ATCATCGTCAAACAAGAGGCCTT		Zapparoli *et al*. (1998)

Table 3.3 Thermal cycling conditions used for the colony PCR of wine LAB.

Primer pair	T_{Di} (°C), time	Main cycling conditions				T_{Ef} (°C), time
		Number of cycles	T_D (°C), time	T_A (°C), time	T_E (°C), time	
planF (fwd) / pREV (rev)	94°C, 5 min	30	94°C, 30 sec	56°C, 10 sec	72°C, 30 sec	72°C, 5 min
On1 (fwd) / On2 (rev)	94°C, 5 min	40	94°C, 1 min	64°C, 45 sec	72°C, 1 min	72°C, 10 min

T_{Di}, initial denaturing temperature; T_D, denaturing temperature; T_A, annealing temperature; T_E, extension temperature; T_{Ef}, final extension temperature

3.2.3 SCREENING IN SYNTHETIC WINE MEDIUM

Growth conditions for bacteria cultures prior to inoculation in the synthetic wine medium were as described previously. The ability of the bacterial strains to degrade malic acid was evaluated in a synthetic wine medium adapted from Ugliano *et al*. (2003). The composition of the synthetic wine medium can be seen in **Table 3.4**.

The media was prepared with the following changes: the pH of the medium was adjusted to 3.4 with potassium hydroxide (KOH) pellets (Saarchem, Merck) and the ethanol concentration was adjusted to 14% (v/v) with 100% ethanol (Saarchem, Merck). No glycoside extract was added and 0.056 g/L $MnSO_4.H_2O$ was added as a replacement for $MnSO_4$. The media was filtered through a 0.45 µm syringe filter (Lasec), followed by filtration through a 0.22 µm syringe filter (Lasec). The pre-cultured bacteria were inoculated at 1.5% (v/v) (at approximately 10^6 cells/mL) in the synthetic wine medium and incubated at 20-22°C under static anaerobic conditions to undergo MLF. Cell counts were monitored on day 0, 2 and 6 of fermentation by plating out on MRS or MRST agar plates and incubating the plates anaerobically at 30°C. The malic acid concentration was determined with a malic acid enzymatic assay kit (Roche, Boehringer Mannheim, Germany) on day seven of MLF.

Table 3.4 The constituents of the synthetic wine medium used for the screening of the LAB strains for the ability to degrade malic acid.

Composition	Concentration
L-(+)-Tartaric acid (Saarchem, Merck)	5.0 g/L
L-(-)-Malic acid (Sigma)	3.5 g/L
Acetic acid (Saarchem, Merck)	0.6 g/L
D-(+)-Glucose (Sigma)	2.0 g/L
D-(-)-Fructose (Sigma)	2.0 g/L
NaCl (Saarchem, Merck)	0.2 g/L
$(NH_4)_2SO_4$ (BOH, Merck)	1.0 g/L
K_2HPO_4 (Fluka)	2.0 g/L
$MgSO_4.7H_2O$ (Saarchem, Merck)	0.2 g/L
$MnSO_4.H_2O$ (Univar, Saarchem)	0.056 g/L

3.2.4 MOLECULAR DETECTION OF BIOGENIC AMINE GENES

3.2.4.1 DNA preparation

The isolation of genomic DNA from *Lb. plantarum* and *O. oeni* were done according to a method adapted from Lewington *et al*. (1987). For DNA preparation purposes, the LAB strains were grown at 30°C in MRS and MRSA broth supplemented with 1.5% (w/v) glycine (Saarchem, Merck) for 24 hours.

Two millilitres of pre-cultured bacteria were harvested for two minutes and re-suspended in 75 µL of 0.25 mol/L sucrose (Saarchem, Merck) and 50 mmol/L Tris-HCl pH 8. Lysozyme (DSM Food Specialties, Oenology, France) was dissolved in milliQ water (Millipore water purification system) and added to the suspension at 30 mg/mL, mixed and left at 37°C for 40 minutes. This was followed with the addition of 50 µL 20% (w/v) sodium dodecyl sulphate (SDS) (Saarchem, Merck) (pre-warmed to 37°C). The suspension was gently mixed until the cells had lysed completely and vortexed for 5 seconds after which 25 µL of ice-cold 5 M NaCl was added and thoroughly mixed. After one hour, the mixture was centrifuged and the supernatant removed to a clean tube after which an equal volume of chloroform/isoamyl alcohol (24:1) was added. The extraction was allowed to continue for 10 minutes, centrifuged for 10 minutes and the supernatant removed. The genomic DNA was precipitated by adding one tenth volume of 3 M sodium acetate at pH 5.2 and two volumes of pre-chilled 100% ethanol. The mixture was left at -20°C for 10 minutes and centrifuged for 5 minutes to harvest genomic DNA. A 100 µL of TE (1 M Tris-HCl, 0.5 M EDTA, pH 8.0) was added to the pellet and the DNA re-precipitated by adding 10 µL sodium acetate and 275 µL prechilled 100% ethanol. The two phases were separated by centrifugation for

10 minutes. The pellet was washed with 70% ethanol, dried in a speedy vacuum and redissolved in 40 µL of milliQ water. The samples were stored at -20°C.

The quantification of DNA was performed spectrophotometrically using a NanoDrop® ND-1000 (NanoDrop Technologies, Inc., Wilmington, USA).

3.2.4.2 PCR detection of genes

Bacteria strains from *Lb. plantarum* and *O. oeni* that successfully degraded malic acid in the synthetic wine medium were screened for the genes that encode for the histidine-, tyrosine- and ornithine decarboxylase enzymes that produce the biogenic amines histamine, tyramine and putrescine, respectively. This was done with a multiplex PCR method described by Marcobal *et al*. (2005). The primer sets for the decarboxylase genes can be seen in **Table 3.5**. The 50 µL reaction mixture contained 100 ng template DNA (section 3.2.4.1), 0.3 µM of primer set JV16HC/JV17HC (Whitehead Scientific, South Africa), 1 µM of primer set 3/16 and 2 µM of primer set P1-rev/P2-for, 200 µM dNTP's (Takara, Seperations), 1.75 mM $MgCl_2$ (Supertherm, Southern Cross Biotechnology), 1x PCR buffer (Supertherm, Southern Cross Biotechnology) and 1.5 U DNA Polymerase (Supertherm, Southern Cross Biotechnology).

Lactobacillus 30a is a histidine- and putrescine-producing LAB strain (Le Jeune *et al*., 1995; Marcobal *et al*., 2005). This strain was used as a positive control for these two gene products during the PCR assay. *Lactobacillus brevis* M58, a tyramine producing LAB strain previously isolated from South African brandy base wine, was selected as a positive control in the PCR reaction (Downing, 2003; Du Plessis *et al*., 2004). A negative control contained no bacterial DNA template. PCR reaction conditions were as described in **Table 3.6**.

All PCR reactions were done using a T3 Thermocycler (Whatman Biometra GmbH, Germany). PCR products were analysed by gel electrophoresis in 1.5% (w/v) agarose (Whitehead Scientific) gels containing ethidium bromide (Sigma-Aldrich). Gels were run at 85V in a 1x TAE buffer (100 mM Tris-Cl, 1 mM EDTA, pH 8.0, 0.20 mM acetate) for approximately 45 minutes. UV transillumination was used for visualising DNA fragments and an Alpha Imager (Alpha Innotech Corporation, San Leandro, California) used for documenting the image. Lambda DNA (Roche) digested with *BstE* II (Roche) was used as the standard molecular weight marker.

Table 3.5 The primer sequences for the detection of the genes associated with biogenic amine formation in wine LAB.

Primer name	Enzyme	Primer sequence (5'-3')
JV16HC	Histidine decarboxylase	AGATGGTATTGTTTCTTATG
JV17HC	Histidine decarboxylase	AGACCATACACCATAACCTT
3	Ornithine decarboxylase	GTNTTYAAYGCNGAYAARACNTAYTTYGT
16	Ornithine decarboxylase	TACRCARAATACTCCNGGNGGRTANGG
P1 - rev	Tyrosine decarboxylase	CCRTARTCNGGNATAGCRAARTCNGTRTG
P2 - fwd	Tyrosine decarboxylase	GAYATNATNGGNATNGGNYTNGAYCARG

Table 3.6 Thermal cycling conditions for the multiplex PCR used in the detection of the genes associated with biogenic amine formation in wine LAB.

	T_{Di} (°C), time	Main cycling conditions				T_{Ef} (°C), time
		Number of cycles	T_D (°C), time	T_A (°C), time	T_E (°C), time	
Multiplex PCR	94°C, 10 min	30	95°C, 30 sec	52°C, 30 sec	72°C, 2 min	72°C, 10 min

T_{Di}, initial denaturing temperature; T_D, denaturing temperature; T_A, annealing temperature; T_E, extension temperature; T_{Ef}, final extension temperature

3.2.5 GENETIC SCREENING OF ENZYMES

The next section of the project focused on the LAB strains that were positively identified as *Lb. plantarum* or *O. oeni*, could successfully degrade malic acid in the synthetic wine medium and did not possess any of the genes associated with biogenic amine formation. The LAB strains were genetically screened for the presence of enzymes associated with aroma production during MLF. The enzymes of interest that were screened for with PCR and gene-specific primers include *β*-glucosidase, PAD, citrate lyase, esterase and protease. The PCR reactions were done with isolated genomic DNA serving as the DNA template and gene-specific primer sets.

3.2.5.1 PCR detection of genes

The PCR detection of the genes associated with the formation and modification of aroma compounds during MLF, were done using genomic DNA and specific primer sets. Primer sets for *β*-glucosidase, PAD and citrate lyase were the same for both *Lb. plantarum* and *O. oeni*, whereas for protease and esterase, individual primer sets existed or were designed for *Lb. plantarum* and *O. oeni*. The gene-specific primer sets are depicted in **Table 3.7**. Gene-specific primers used for PCR amplification were designed from nucleotide sequences coding for the esterase and protease genes in *O. oeni*. The gene nucleotide sequences retrieved from the National Center for

Biotechnology Information (NCBI) were used to design enzyme-specific amplification primers for the detection of the different enzyme genes from *O. oeni*. *Oenococcus oeni* strain PSU-1 was used as the basis for designing primers for amplifying the coding regions of a predicted esterase and a trypsin-like serine protease. Primer synthesis was done by Whitehead Scientific, Cape Town, South Africa. Strains from the IWBT culture collection that were screened in a previous study by Mtshali (2007) and were positive for the respective enzyme genes were used as positive controls in the PCR reactions (**Table 3.7**). A negative control contained no bacterial DNA template.

The PCR reaction mixture for the detection of the gene encoding for the *β*-glucosidase enzyme in *Lb. plantarum* and *O. oeni*, contained the following: a 50 µL reaction mixture with 100 ng template DNA, 0.5 µM of each primer in the primer set BGL-1/BGL-2 (Whitehead Scientific, South Africa), 200 µM dNTP's (Takara, Seperations), 1.5 mM $MgCl_2$ (Supertherm, Southern Cross Biotechnology), 1x PCR buffer (Supertherm, Southern Cross Biotechnology) and 1.25 U DNA Polymerase (Supertherm, Southern Cross Biotechnology).

The PCR reaction mixture for the detection of the gene encoding for PAD in *Lb. plantarum* and *O. oeni* consisted of the following: a 50 µL reaction mixture with 100 ng template DNA, 0.4 µM of each primer in the primer set PAD-1/PAD-3, 200 µM dNTP's, 1x PCR buffer and 1 U ExTaq DNA Polymerase (Takara Biomedicals).

The PCR reaction mixture for the detection of the gene encoding for citrate lyase in *Lb. plantarum* and *O. oeni* contained the following: a 25 µL reaction mixture with 100 ng template DNA, 0.4 µM of each primer in the primer set Clase-1/Clase-2, 250 µM dNTP's, 0.75 mM $MgCl_2$, 1x PCR buffer and 2 U Supertherm Taq DNA Polymerase (Southern Cross Biotechnology).

Table 3.7 Nucleotide sequences of primer sets used for the genetic screening of enzymes in *Lb. plantarum* and *O. oeni.*

Primer name	Primer sequence (5'-3')	Organism	Positive controls	Application	Reference
BGL-1	GTGACTATGGTAGAGTTTCC - fwd	*O. oeni* *Lb. plantarum*	*Lb. plantarum* 14.1 *Lb. plantarum* 76.2	*β*-glucosidase gene	Spano *et al*. (2005)
BGL-2	TCAAAACCCATTCCGTTCCCCA - rev				
PAD-1	AARAAYGAYCAYACYRTTGATTACC - fwd	*O. oeni* *Lb. plantarum*	*Lb. plantarum* 14.1 *Lb. plantarum* 66.1	Phenolic acid decarboxylase gene	Mtshali (2008)
PAD-3	TTCTTCWACCCAYTTHGGGAAGAA - rev				
Clase-1	TTACGBCGSACRATGATGTTTGT -fwd	*O. oeni* *Lb. plantarum*	*Lb. plantarum* 14.1 *Lb. plantarum* 66.1	Citrate lyase gene	Mtshali (2008)
Clase-2	TATTTTTCAATGTAATTDCCCTCC - rev				
Est-1	GCTAATTTGTAACCGTATCCGCC - fwd	*Lb. plantarum*	*Lb. plantarum* 14.1 *Lb. plantarum* 76.2	Putative esterase gene	Mtshali (2007)
Est-2	CGCGCATGTTAACTTTTAGTAGAAC - rev				
Est-O-1	ATGGCATTTTTAGAAGTTAATTATTATTCACG - fwd	*O. oeni*	*O. oeni* 1098	Predicted esterase	This work
Est-O-2	CTATGACAAACGTTTTTCTGCTTGATAATT - rev				
Prt-1	GCATGGCTAATAAATCATTAATCAAAG - fwd	*Lb. plantarum*	*Lb. plantarum* 14.1 *Lb. plantarum* 76.2	Serine protease HtrA gene	Mtshali (2007)
Prt-2	GCTTAGTTACTTTGTTTAGTTAACGTTTTG - rev				
Prt-O-1	GTGACTGAAGAACAAGACCAAGGAAAAAC - fwd	*O. oeni*	*O. oeni* 1098	Trypsin-like serine protease	This work
Prt-O-2	TTATTGTTTCAAAGTTTCAGTCATCTTAACCTT - rev				

The PCR reaction mixture for the detection of the genes encoding for esterase and protease in *Lb. plantarum* contained the following: a 50 µL reaction mixture with 100 ng template DNA, 0.4 µM of each primer in the primer sets Est-1/Est-2 for esterase detection and Prt-1/Prt-2 for protease detection, 250 µM dNTP's, 1.5 mM $MgCl_2$, 1x PCR buffer and 0.025 U Supertherm Taq DNA Polymerase.

The PCR reaction mixture for the detection of the genes encoding for esterase and protease in *O. oeni* consisted of the following: a 50 µL reaction mixture with 100 ng template DNA, 2 µM of each primer in the primer sets Est-O-1/Est-O-2 for esterase detection and Prt-O-1/Prt-O-2 for protease detection, 250 µM dNTP's, 1.5 mM $MgCl_2$, 1x PCR buffer and 1.25 U Supertherm Taq DNA Polymerase.

All PCR reactions were done using a T3 Thermocycler (Whatman Biometra GmbH, Germany) through the reaction conditions as described in **Table 3.8**. PCR products were analysed by gel electrophoresis in 1-3% (w/v) agarose (Whitehead Scientific) gels containing ethidium bromide (Sigma-Aldrich). Gels were run at 85V in a 1x TAE buffer (100 mM Tris-Cl, 1 mM EDTA, pH 8.0, 0.20 mM acetate) for approximately 45-60 minutes. UV transillumination was used for visualising DNA fragments and an Alpha Imager (Alpha Innotech Corporation, San Leandro, California) used for documenting the image. DNA Molecular Weight Marker XIV (Roche) was used as the standard molecular weight marker.

Table 3.8 Thermal cycling conditions of the PCR reactions used for the genetic screening of the enzymatic profiles in wine LAB.

Primer pair	T_{Di} (°C), time	Main cycling conditions				T_{Ef} (°C), time
		Number of cycles	T_D (°C), time	T_A (°C), time	T_E (°C), time	
BGL-1/BGL-2	94°C, 5 min	30	94°C, 1 min	50°C, 40 sec	72°C, 62 sec	72°C, 10 min
PAD-1/PAD-3	94°C, 2 min	35	94°C, 40 sec	50°C, 1 min	72°C, 30 sec	72°C, 5 min
Clase-1/Clase-2	94°C, 3 min	35	94°C, 30 sec	54°C, 1 min	72°C, 1 min	72°C, 10 min
Est-1/Est-2	94°C, 5 min	30	94°C, 1 min	53°C, 30 sec	72°C, 1 min	72°C, 10 min
Est-O-1/Est-O-2	94°C, 5 min	35	94°C, 1 min	50°C, 1 min	72°C, 1 min	72°C, 10 min
Prt-1/Prt-2	94°C, 5 min	30	94°C, 1 min	55°C, 30 sec	72°C, 1 min	72°C, 10 min
Prt-O-1/Prt-O-2	94°C, 5 min	35	94°C, 1 min	52°C, 90 sec	72°C, 90 sec	72°C, 10 min

T_{Di}, initial denaturing temperature; T_D, denaturing temperature; T_A, annealing temperature; T_E, extension temperature; T_{Ef}, final extension temperature

3.3 RESULTS AND DISCUSSION

3.3.1 IDENTIFICATION OF LACTIC ACID BACTERIA

LAB from the IWBT culture collection and isolated from spontaneous MLF, were identified as either *Lb. plantarum* or *O. oeni*. The strains were identified using colony PCR and species-specific primers. Only the strains selected for this study that were able to proliferate under the culture conditions, were selected for colony PCR.

The species-specific primers amplified single products of 319 base pairs (bp) for *Lb. plantarum* and 1025 bp for *O. oeni*. **Figures 3.1** and **3.2** show an example of the amplification products obtained using the species-specific primer sets for *Lb. plantarum* and *O. oeni*, respectively. A total of 23 strains were positively identified as *O. oeni*, including 13 of the 15 strains (87%) isolated from spontaneous MLF. This is to be expected as *O. oeni* is the predominant LAB present in wine. Nineteen strains were positively identified as *Lb. plantarum*. The identified strains are listed in **Table 3.1**.

The 42 strains of LAB that were identified as *O. oeni* or *Lb. plantarum* were selected for the second phase of the screening process and evaluated in the synthetic wine medium for their ability to degrade malic acid.

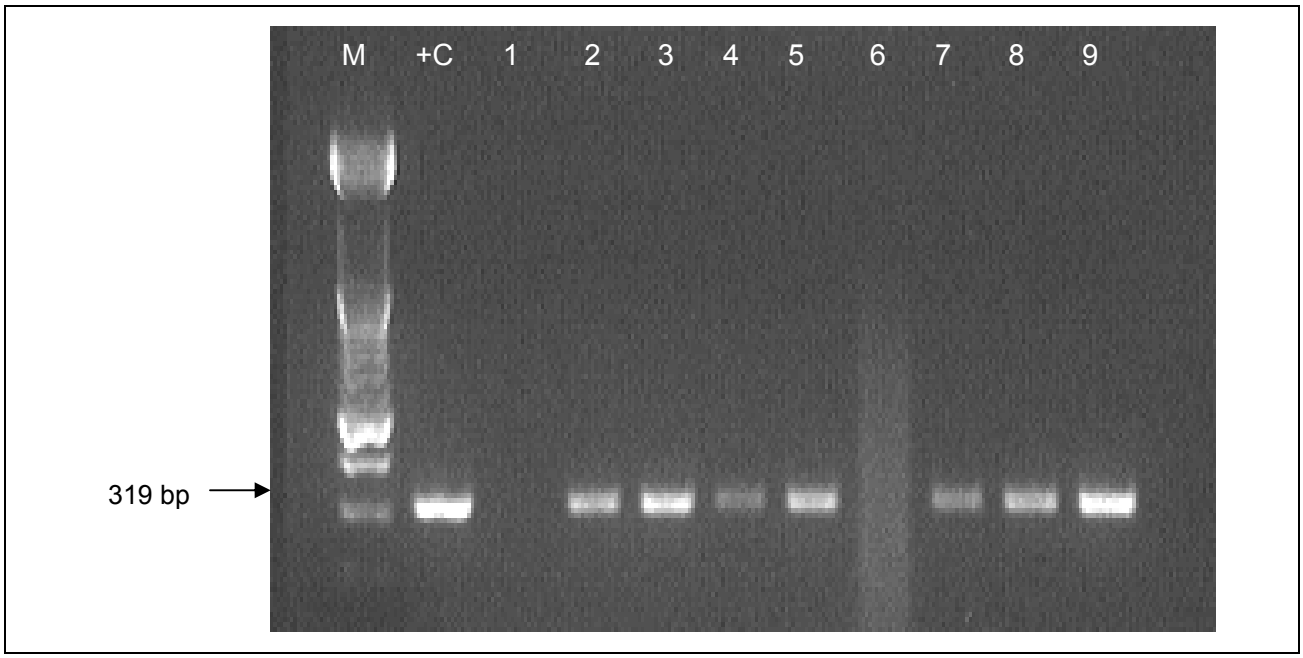

Figure 3.1 Agarose gel of the PCR amplification products obtained using the *Lb. plantarum* species-specific primer set. Lane M: 100bp DNA Molecular Weight Marker XIV. Lane +C: *Lb. plantarum* reference strain (positive control). The LAB isolates (by lane number) tested: 1, negative control; 2, Culture 14.1; 3, Culture 56; 4, Culture 66.1; 5, Culture 68; 6, Culture 82; 7, Culture 71.1; 8, Culture 78.1; 9, Culture 107.

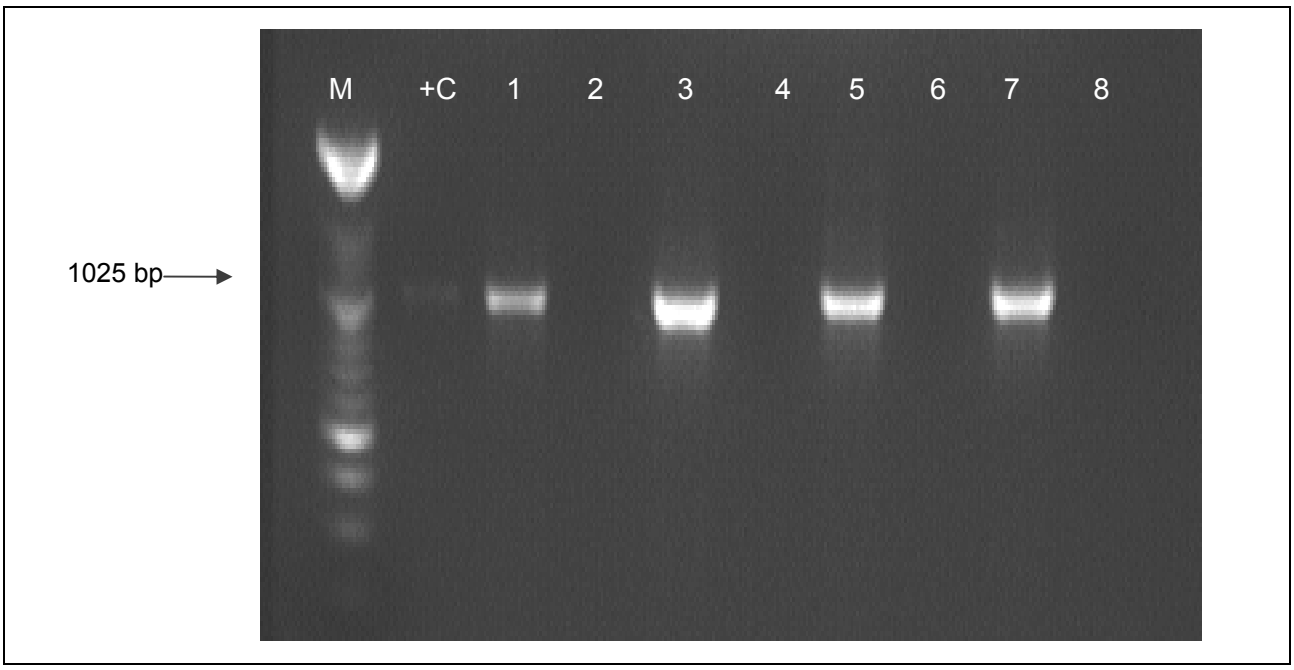

Figure 3.2 Agarose gel of the PCR amplification products obtained using the *O. oeni* species-specific primer set. Lane M: 100bp DNA Molecular Weight Marker XIV. Lane +C: Lalvin VP41 (Lallemand) (positive control). The LAB isolates (by lane number) tested: 1, *O. oeni* 1098 (positive control); 2, negative control; 3, Culture A1; 4, Culture A3; 5, Culture B1; 6, Culture B2; 7, Culture C1; 8, Culture C2.

3.3.2 SCREENING IN SYNTHETIC WINE MEDIUM

The screening process in the synthetic wine medium was used as an indication of the possible survival and performance of the strains in the actual wine environment. **Table 3.9** shows the LAB strains and the corresponding malic acid concentrations measured in the synthetic wine medium after seven days of MLF at 20-22°C.

The *Lb. plantarum* strains performed the best and seven of the strains were able to convert the initial malic acid concentration of 3.5 g/L to below 0.3 g/L in seven days. These seven *Lb. plantarum* strains were isolates 14.1, 56, 66.1, 68, 71.1, 78.1 and 107. The seven *O. oeni* strains that showed the most potential in the synthetic medium, combined with the results obtained by the monitoring of the cell numbers for the duration of MLF (results not shown), were selected for further use in the study. These were *O. oeni* cultures A2, B1, S5, S6, E53, J65 and W56. The results obtained in the synthetic wine medium are only indicative of the potential performance of the LAB strains in the actual wine environment. However, the biggest limitation of the synthetic wine medium is the lack of phenolic compounds in the medium. LAB are sensitive to various phenolic compounds present in wine (Stead, 1993; Vivas *et al*., 1997; Reguant *et al*., 2000; Hernández *et al*., 2006; 2007; García-Ruiz *et al*., 2008) and a medium that include phenolic compounds would give a better indication of the eventual performance of the strains in wine.

Table 3.9 The malic acid concentration measured after seven days in the synthetic wine medium inoculated with the different LAB strains. The synthetic wine medium had an initial malic acid concentration of 3.5 g/L.

LAB	Malic acid (g/L)	LAB	Malic acid (g/L)
O. oeni B69	1.08	*Lb. plantarum* 2.1	0.71
O. oeni D59	0.73	*Lb. plantarum* 14	0.83
O. oeni D60	0.28	*Lb. plantarum* 14.1	0.00
O. oeni E53	0.28	*Lb. plantarum* 56	0.00
O. oeni J65	0.81	*Lb. plantarum* 65	1.32
O. oeni M69	1.08	*Lb. plantarum* 66.1	0.27
O. oeni N73	0.10	*Lb. plantarum* 68	0.02
O. oeni W56	1.09	*Lb. plantarum* 69	0.17
O. oeni W75	1.11	*Lb. plantarum* 70	0.08
O. oeni W77	0.98	*Lb. plantarum* 71	0.72
O. oeni A1	0.98	*Lb. plantarum* 71.1	0.17
O. oeni A2	0.58	*Lb. plantarum* 73.1	0.41
O. oeni B1	0.49	*Lb. plantarum* 75	1.08
O. oeni C1	1.26	*Lb. plantarum* 76.2	0.53
O. oeni S1	0.49	*Lb. plantarum* 77.1	1.10
O. oeni S2	0.35	*Lb. plantarum* 78.1	0.24
O. oeni S3	0.95	*Lb. plantarum* 80.2	1.04
O. oeni S4	0.28	*Lb. plantarum* 107	0.05
O. oeni S5	0.25	*Lb. plantarum* 109	0.65
O. oeni S6	0.32		
O. oeni S7	0.55		
O. oeni S8	0.40		
O. oeni S9	1.05		

3.3.3 BIOGENIC AMINE GENES

The seven *Lb. plantarum* and seven *O. oeni* strains that successfully degraded malic acid in the synthetic wine medium were screened for the genes that encode for the amino acid decarboxylase enzymes responsible for biogenic amine formation. The primers amplified single products of 367 bp and 1446 bp for *Lactobacillus* 30a, corresponding to the histidine- and ornithine decarboxylase (HDC and ODC) enzymes respectively, and a product of 924 bp for *Lb. brevis* M58, corresponding to the tyramine decarboxylase (TDC) enzyme. Of the 14 strains that were screened (all results not shown), none contained HDC, ODC or TDC genes (**Figure 3.3**).

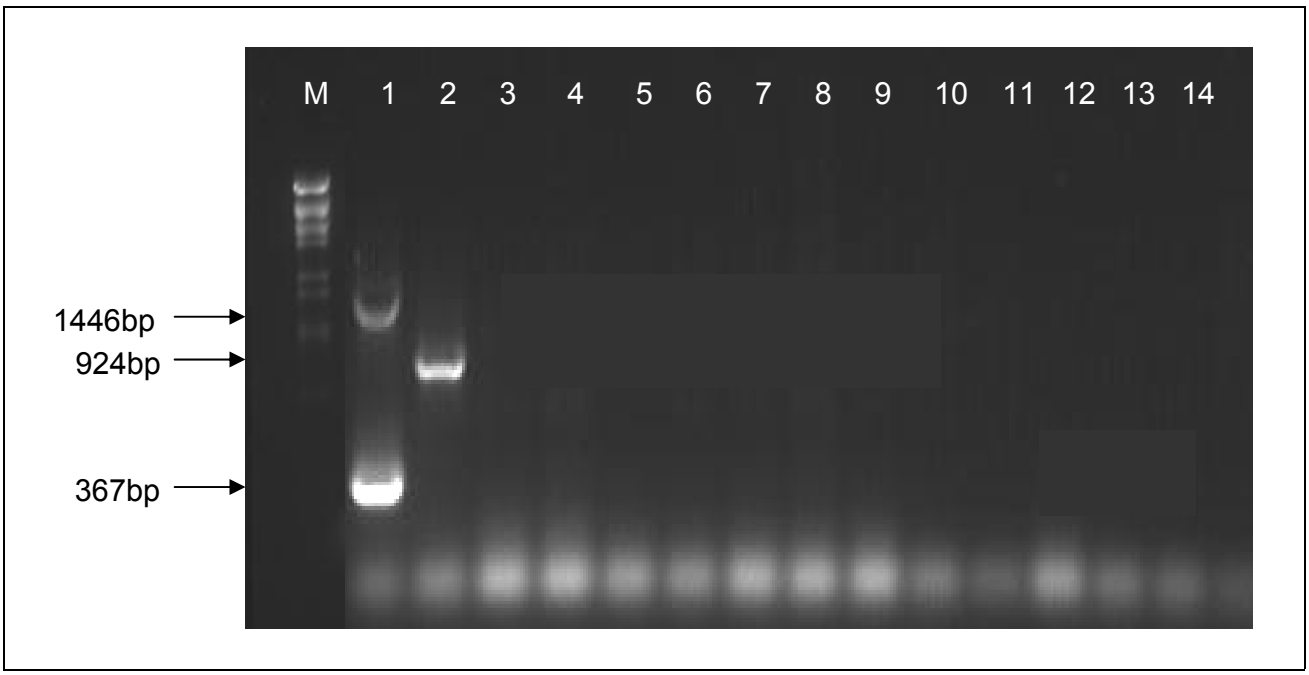

Figure 3.3 Agarose gel of the PCR amplification products obtained for *Lb. plantarum* and *O. oeni* using the specific primer sets for the detection of biogenic amine encoding genes. Lanes: M, Lambda DNA *BstE* II marker; 1, *Lactobacillus* 30a (reference strain for ODC and HDC); 2, *Lb. brevis* M58 (IWBT culture collection) (positive control for TDC); 3, control PCR reaction without any DNA template; 4, *Lb. plantarum* 14.1; 5, *Lb. plantarum* 56; 6, *Lb. plantarum* 66.1; 7, *Lb. plantarum* 68; 8, *Lb. plantarum* 71.1; 9, *Lb. plantarum* 78.1; 10, *Lb. plantarum* 107; 11, *O. oeni* A2; 12,*O. oeni* B1; 13, *O. oeni* S5; 14, *O. oeni* S6.

The inability to produce biogenic amines is an important characteristic for any strain that is being considered for use in a starter culture, as biogenic amines have an impact on wine wholesomeness and have several health implications as well as impacting on the wine aroma.

3.3.4 ENZYMATIC PROFILES

Genetic screening of the enzymatic profiles were done for the strains that were positively identified as *O. oeni* or *Lb. plantarum*, could degrade malic acid in the synthetic wine medium and were negative for the genes associated with biogenic amine formation. The enzymes of interest that could possibly influence the aroma profile of the wine and that were screened for, included *β*-glucosidase, PAD, citrate lyase, esterase and protease.

Figures 3.4 to **3.9** show some of the screening results obtained for the enzymes evaluated in the LAB strains. The primers amplified single products of 219 bp for PAD (**Figure 3.4**), 897 bp for citrate lyase (**Figure 3.5**), 1278 bp for protease in *O. oeni* (**Figure 3.6**), 804 bp for esterase in *O. oeni* (**Figure 3.7**) and 1392 bp for *β*-glucosidase (**Figures 3.8** and **3.9**).

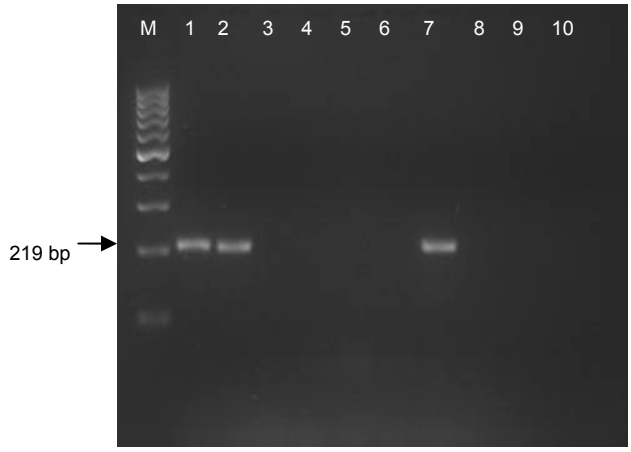

Figure 3.4 Agarose gel for *Lb. plantarum* and *O. oeni* of the PCR amplification products obtained using the specific primer sets for PAD. Lanes: M, 100bp DNA Molecular Weight Marker XIV; 1, *Lb. plantarum* 66.1 (positive control); 2, *Lb. plantarum* 14.1 (positive control); 3, negative control; 4, *O. oeni* E53; 5, *O. oeni* J65; 6, *O. oeni* W56; 7, *Lb. plantarum* 71.1; 8, *O. oeni* A2; 9, *O. oeni* B1; 10, *O. oeni* S1.

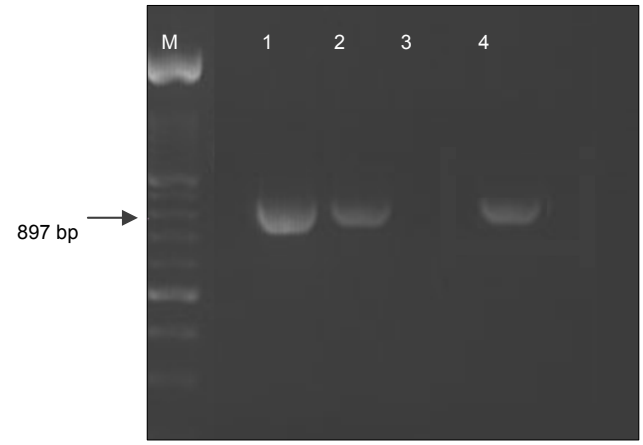

Figure 3.5 Agarose gel for *Lb. plantarum* of the PCR amplification products obtained using the specific primer sets for citrate lyase. Lanes: M, 100bp DNA Molecular Weight Marker XIV; 1, *Lb. plantarum* 14.1 (positive control); 2, *Lb. plantarum* 66.1 (positive control); 3, negative control; 4, *Lb. plantarum* 68.

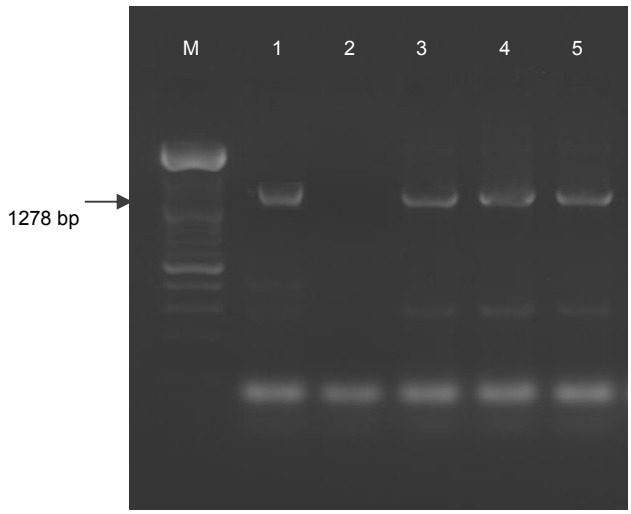

Figure 3.6 Agarose gel for *O. oeni* of the PCR amplification products obtained using the specific primer sets for protease. Lanes: M, 100bp DNA Molecular Weight Marker XIV; 1, *O. oeni* 1098 (positive control); 2, negative control; 3, *O. oeni* E53; 4, *O. oeni* J65; 5, *O. oeni* W56.

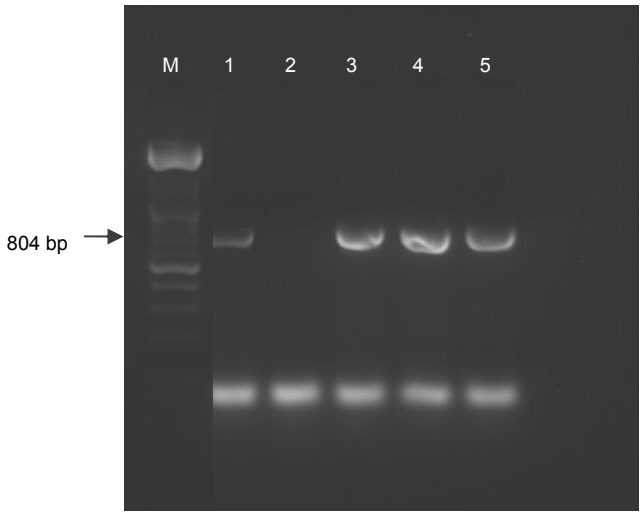

Figure 3.7 Agarose gel for *O. oeni* of the PCR amplification products obtained using the specific primer sets for esterase. Lanes: M, 100bp DNA Molecular Weight Marker XIV; 1, *O. oeni* 1098 (positive control); 2, negative control; 3, *O. oeni* E53; 4, *O. oeni* J65; 5, *O. oeni* W56.

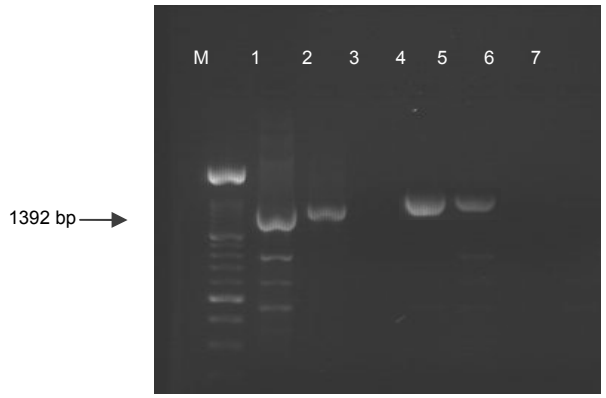

Figure 3.8 Agarose gel for *Lb. plantarum* of the PCR amplification products obtained using the specific primer sets for *β*-glucosidase. Lanes: M, 100bp DNA Molecular Weight Marker XIV; 1, *Lb. plantarum* 14.1 (positive control); 2, *Lb. plantarum* 76.2 (positive control); 3, negative control; 4, *Lb. plantarum* 56; 5, *Lb. plantarum* 66.1; 6, *Lb. plantarum* 78.1; 7, *Lb. plantarum* 107.

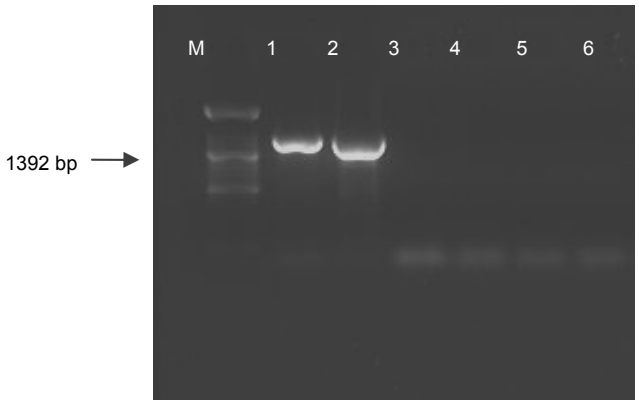

Figure 3.9 Agarose gel for *O. oeni* of the PCR amplification products obtained using the specific primer sets for *β*-glucosidase. Lanes: M, 100bp DNA Molecular Weight Marker XIV; 1, *Lb. plantarum* 76.2 (positive control); 2, *Lb. plantarum* 14.1 (positive control); 3, negative control; 4, *O. oeni* E53; 5, *O. oeni* J65; 6, *O. oeni* W56.

All seven *Lb. plantarum* strains tested positive for the presence of the citrate lyase gene and the predicted esterase gene. Six of the strains tested positive for *β*-glucosidase, protease and PAD (**Table 3.10**). The seven *O. oeni* strains tested positive for the citrate lyase gene. All the strains were negative for PAD and *β*-glucosidase, but positive for predicted esterase and trypsin-like serine protease (**Table 3.11**).

Table 3.10 The results from the genetic screening of the enzymes of the *Lb. plantarum* strains. Presence of the gene is indicated with (+) and absence of the gene is indicated with (-).

***Lb. plantarum* strains**	***β*-glucosidase**	**PAD**	**Citrate lyase**	**Esterase**	**Protease**
14.1	+	+	+	+	+
56	+	+	+	+	+
66.1	+	+	+	+	+
68	+	+	+	+	+
71.1	+	+	+	+	-
78.1	-	-	+	+	+
107	+	+	+	+	+

Table 3.11 The results from the genetic screening of the enzymes of the *O. oeni* strains. Presence of the gene is indicated with (+) and absence of the gene is indicated with (-).

***O. oeni* strains**	***β*-glucosidase**	**PAD**	**Citrate lyase**	**Esterase**	**Protease**
A2	-	-	+	+	+
B1	-	-	+	+	+
S5	-	-	+	+	+
S6	-	-	+	+	+
E53	-	-	+	+	+
J65	-	-	+	+	+
W56	-	-	+	+	+

The biggest differences in the enzymatic profiles of *O. oeni* and *Lb. plantarum* are the presence/absence of the *β*-glucosidase gene and the *pad* gene. However, these PCR reactions only confirm the presence of these genes; it does not give an indication of the activity, if any, of these genes under wine conditions. The results of enzymatic screenings for the *β*-glucosidase gene reported in literature, are contradictory, with some studies reporting activity of the gene in synthetic medium (Guilloux-Benatier *et al*., 1993), whilst others found no enzymatic activity against native grape glycosides (Mansfield *et al*., 2002).

Six of the seven (86%) *Lb. plantarum* strains have the potential to release glycosidically bound flavour compounds, compared to none of the *O. oeni* strains possessing the *β*-glucosidase gene. These findings are in agreement with results by Mtshali (2007), who also found the absence of the *β*-glucosidase gene in the *O. oeni* strains screened in that study. Similarly, six of the seven *Lb. plantarum* strains have the potential to produce volatile phenols, compared to the absence of the PAD gene in all of the *O. oeni* strains. These results show that the *Lb. plantarum* strains have a more complex enzymatic profile and therefore have the ability to contribute to the aroma profile during MLF. The strains screening positive for the presence of the citrate lyase, esterase and protease genes, could potentially contribute to the aroma profile of wine and add complexity via the production of diacetyl, esters and nitrogen containing compounds, respectively.

3.4 CONCLUSIONS

A total of 52 LAB strains were selected for characterisation and possible use as a starter culture for MLF. Thirteen strains, which had been previously identified as *O. oeni* and 24 strains, which had been previously identified as *Lb. plantarum*, were selected from the IWBT culture collection. Fifteen

strains isolated from spontaneous MLF in Pinotage from the Paarl region, were also included in the study.

Of these 52 strains, the identity of 23 strains were confirmed as *O. oeni* (including 13 of the strains isolated from spontaneous MLF) and 19 strains as *Lb. plantarum*, using colony PCR and species-specific primers. These strains were then evaluated in a synthetic wine medium for their ability to degrade malic acid. The 14 strains that showed the most potential in the synthetic wine medium, seven *O. oeni* and seven *Lb. plantarum*, were selected for further characterisation. A multiplex-PCR method was used to screen for the presence of genes encoding for the enzymes responsible for biogenic amine formation and none of the 14 strains contained any of these genes. These 14 strains were genetically screened for enzymes pertaining to the aroma profile of wine undergoing MLF. The *Lb. plantarum* strains showed a more complex enzymatic profile compared to that of the *O. oeni* strains, with the biggest differences relating to the *β*-glucosidase and *pad* genes.

These strains will have to be evaluated in small-scale wine fermentations to investigate their ability to survive in the wine environment.

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Chapter 4

Research results

Small-scale fermentations with characterised lactic acid bacteria to assess the influence on aroma compounds and sensory evaluation of the wine

This manuscript will be submitted for publication in
International Journal of Food Microbiology

4. RESEARCH RESULTS

Small-scale fermentations with characterised LAB to assess the influence on aroma compounds and sensory evaluation of the wine

4.1 INTRODUCTION

Malolactic fermentation (MLF) is an intricate process, usually following the completion of alcoholic fermentation (AF) by the yeast, and is a result of the metabolic processes associated with the presence and metabolism of lactic acid bacteria (LAB) (Wibowo *et al*., 1985; Lonvaud-Funel, 1999; Ugliano and Moio, 2005; Jussier *et al*., 2006). The three main reasons for conducting MLF is the conversion of L-malic acid to L-lactic acid, the removal of malic acid to ensure microbial stability and the production of a wanted aroma profile according to the required style of wine (Davis *et al*., 1988; Kunkee, 1991; Maicas *et al*., 1999; Liu 2002; Ugliano *et al*., 2003). The indigenous LAB associated with the wine environment usually belong to the genera of *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc*, while commercially available MLF starter cultures comprise of only *Oenococcus oeni* strains (Wibowo *et al*., 1985; Lonvaud-Funel, 1999).

It is becoming clear that the practice of inoculating for MLF has various advantages, including increased chances of the successful completion of MLF and reduced risks associated with spontaneous MLF by spoilage LAB. Risks associated with spontaneous MLF include the formation of biogenic amines and ethyl carbamate, increased volatile acidity concentrations, as well as the possible production of unpleasant flavour compounds like polysaccharides and nitrogen containing compounds responsible for ropiness and mousy off-flavours, respectively (Davis *et al*., 1985; Fugelsang and Zoecklein, 1993; Henick-Kling, 1995; Bartowsky and Henschke, 1995; Lonvaud-Funel, 1999). The changes associated with the presence of LAB and MLF are largely strain dependant and therefore the selection, screening and characterisation of isolates for use as a starter culture is essential (Britz and Tracey, 1990; Henick-Kling, 1993). There are various important criteria to consider when selecting cultures for possible use as a MLF starter culture. These include the following: the ability to tolerate low pH, high ethanol and sulphur dioxide (SO_2) concentrations, good growth characteristics under winemaking conditions, compatibility with the selected yeast strain, the inability to produce biogenic amines with a screening for the presence of amino acid decarboxylase genes and the lack of off-flavour or off-odour production (Wibowo *et al*., 1985; Kunkee, 1991; Fugelsang and Zoecklein, 1993; Henick-Kling, 1993; Le Jeune *et al*., 1995; Drici-Cachon *et al*., 1996; Lonvaud-Funel, 2001; Marcobal *et al*., 2004; Volschenk *et al*., 2006).

It is essential to evaluate the influence of various factors on the selected and screened cultures. Factors including ethanol, pH, temperature and SO_2, will have a direct effect in the ability of the LAB culture to survive in the wine environment and complete MLF (Kunkee, 1991; Vaillant *et al*., 1995). Small-scale vinifications therefore play an integral role in evaluating possible cultures

under winemaking conditions (Bou and Powell, 2006). These influencing factors do not only affect the growth ability and the malolactic activity of LAB, but also influence the effect that the LAB cultures will have on the wine aroma.

In addition to evaluating the fermentation capabilities of possible isolates, LAB cultures also have a profound effect on the aroma profile of the wine. LAB are able to modify wine aroma and flavour by metabolising grape constituents, modifying grape- or yeast-derived secondary metabolites and by adsorbing flavour compounds to the cell wall (Bartowsky and Henschke, 1995). Herjavec *et al*. (2001) and Jeromel *et al*. (2008) found wines that had undergone MLF were preferred based on their sensorial profiles, to wines that had not undergone MLF or had undergone spontaneous MLF. Possible aroma impact compounds include diacetyl, esters, volatile fatty acids and higher alcohols (Davis *et al*., 1985; Nielsen and Richelieu, 1999; Maicas *et al*., 1999; Gámbaro *et al*., 2001; Bartowsky *et al*., 2002; Bartowsky and Henschke, 2004; D'Incecco *et al*., 2004; Swiegers *et al*., 2005). Diacetyl is considered to be one of the most important aroma compounds produced by LAB (Bartowsky and Henschke, 1995; Lonvaud-Funel, 1999). This compound is associated with buttery, nutty and butterscotch characters in wine (Bartowsky and Henschke, 1995; 2004; Martineau *et al*., 1995; Bartowsky *et al*., 2002).

Esters are important in determining wine aroma and are responsible for the fruity aromas in young wines. MLF and wine LAB have the ability to alter the ester content of wine (Matthews *et al*., 2004). The extent of this alteration is still unclear, with both increase and decrease in ester concentrations being observed in the literature. The most important esters that typically play a role in MLF are ethyl lactate and diethyl succinate (Maicas *et al*., 1999; Herjavec *et al*., 2001; Ugliano and Moio, 2005). Volatile fatty acids and higher alcohols also contribute to the final wine aroma upon completion of MLF. Lower concentrations of these compounds tend to be beneficial in adding to the complexity of the final wine aroma (Swiegers *et al*., 2005).

It is clear that there are various factors and criteria to consider during the selection of LAB cultures for possible use as a MLF starter culture. The overall objective of this study was to evaluate the previously characterised LAB strains under different winemaking conditions for their potential as MLF starter cultures. The first objective was to assess the MLF rate of the individual *O. oeni* and *Lactobacillus plantarum* strains, followed by the evaluation of the mixed LAB cultures. The fermentation rates were assessed for co-inoculation and sequential inoculation by monitoring malic acid degradation. The second aim was to evaluate the impact of the mixed cultures on the volatile aroma compounds through gas chromatography and the carbonyl compounds by using gas chromatography-mass spectrometry. The effects of the mixed cultures on the sensory profile of the wines were investigated with an informal sensorial evaluation. The datasets were analysed with multivariate data analysis tools. This is the first study on mixed MLF starter cultures to our knowledge.

4.2 MATERIALS AND METHODS

4.2.1 SMALL-SCALE VINIFICATION PROCEDURES AND MICROBIOLOGY

4.2.1.1 Vinification procedures, malolactic fermentation treatments and sampling

Vinifications were conducted in Pinotage, Cabernet Sauvignon and Shiraz in the 2008 season and in Pinotage, Cabernet Sauvignon and Chardonnay in the 2009 season. The grapes were sourced from the Stellenbosch and Wellington regions, South Africa. MLF treatments with individual isolates were done in duplicate in 2008, while the mixed culture MLF treatments were conducted in triplicate in both vintages. Two inoculation scenarios were evaluated in the course of this study: sequential inoculation, where LAB were inoculated after the completion of AF, and co-inoculation, where inoculation for MLF occurred 24 hours after inoculation with the yeast starter culture. Treatments in the 2008 and 2009 vintages were evaluated in sequential inoculation and treatments in the 2009 vintage were evaluated using co-inoculation.

Half a ton of grapes for all vinifications of each cultivar were crushed and destemmed to evaluate the sequential inoculated treatments. Before the onset of AF, representative homogenous samples of the different grape musts were taken to determine the standard wine parameters. SO_2 was added to the must at a concentration of 30 ppm before the onset of AF to inhibit the growth of indigenous microflora. Lysozyme (DSM Food Specialties, Oenology, France) was added at 0.25 g/L to inhibit indigenous LAB. The white grape must had 24 hours of skin contact before being pressed with a hydraulic basket press and being allowed to settle overnight. The red grape must was inoculated for batch AF with a commercial strain of *Saccharomyces cerevisiae*, namely WE372 (active dried yeast) from Anchor Yeast and the white grape must with *S. cerevisiae* VIN2000 (Anchor Yeast). Rehydration and inoculation of the yeast strain was performed according to the manufacturer's specification. AF of the red wine was conducted on the skins. The decrease in sugar levels was measured daily by using a Brix hydrometer to monitor the progression of AF. The fermentation was conducted at 25ºC and the skins were mixed with the juice daily using a manual punch-down method. On the second day of fermentation, Nutrivin (Anchor Yeast), a nutrient supplement was added at 0.5 to 0.7 g/L, depending on the initial quality of the grapes. This aided as a nutrient source for the yeast to avoid stuck or sluggish AF. After AF was completed (less than 1 g/L residual sugar) the red wines were pressed with a hydraulic basket press and divided into the different MLF treatments in 2 L or 4.5 L glass bottles (sealed with airlocks) to complete spontaneous or induced MLF at 20°C.

The wines from the 2008 vintage completed AF and afterwards they were treated with dimethyl dicarbonate (Merck), also known as Velcorin®, at 200 ppm. This completely sterilised the wines before being stored at 4°C for use after the completion of the characterisation stage of the study.

Pinotage, Cabernet Sauvignon and Chardonnay were crushed and destemmed in the 2009 season for evaluating co-inoculation, after which the skins and free-run juice were separated and homogenised. Equal volumes of the homogenised free-run juice and equal weights of the homogenised skins were aliquoted to each treatment (approximately 9 kg) in 10 L buckets. SO_2 was added at a concentration of 20 ppm before the onset of AF. No lysozyme was added to the wines used for co-inoculation evaluation. The individual red grape must treatments were inoculated with *S. cerevisiae* WE372 (Anchor Yeast) and the Chardonnay must with *S. cerevisiae* VIN2000, at the recommended manufacturer's dosage and AF was allowed to commence for 24 hours after which the different MLF treatments were inoculated. Fermentation parameters and monitoring were as described in the previous paragraph with one exception. Co-inoculation wines were moved after the completion of AF from 23°C to 20°C to complete MLF.

4.2.1.2 Malolactic fermentation procedures

A summary of the MLF procedures conducted during the course of this study is shown in **Table 4.1**. The MLF treatments evaluating the individual LAB isolates in sequential inoculation (**Table 4.2**) were conducted in Pinotage in 2008. Based on the performance of the individual strains, certain strains were selected for evaluation in the mixed LAB cultures (**Table 4.3**). The MLF treatments that evaluated the mixture of LAB isolates were performed in sequential inoculation in Pinotage, Cabernet Sauvignon and Shiraz in 2008. Combinations selected based on the 2008 results, were evaluated in both co-inoculation and sequential inoculation conditions in 2009 (**Table 4.4**) in Pinotage, Cabernet Sauvignon and Chardonnay. Descriptions of the treatments are given in **Tables 4.1 to 4.4** and for each vintage separately.

Table 4.1 A summary of the MLF scenarios, bacteria and cultivars evaluated during the course of the study over two vintages.

Cultivar	Vintage	MLF Treatments	Inoculation scenario
Pinotage	2008	Individual isolates [1]	sequential
Pinotage	2008	Mixed isolates [2]	
Cabernet Sauvignon	2008	Mixed isolates	
Shiraz	2008	Mixed isolates	
Pinotage	2009	Mixed isolates [3]	sequential and co-inoculation
Cabernet Sauvignon	2009	Mixed isolates	
Chardonnay	2009	Mixed isolates	

[1] see **Table 4.2** for a description of the MLF treatments with the individual isolates
[2] see **Table 4.3** for a description of the MLF treatments with the mixed cultures in 2008
[3] see **Table 4.4** for a description of the MLF treatments with the mixed cultures in 2009

Several control fermentations were implemented during the course of the study. A commercial malolactic starter culture, Lalvin VP41 from Lallemand, was used as control fermentation in both vintages and for both the individual and mixture of LAB treatments. ML01 (Warrenchem), a malolactic wine yeast, was also used as a control fermentation in the co-inoculation treatments.

These treatments were included for comparing the fermentation performance of the selected LAB with that of commercial products. A treatment receiving no inoculation was used as a spontaneous fermentation control. All commercial yeast and MLF starter cultures added to the wine during small-scale vinifications were inoculated according to the instructions of the manufacturer at the maximum recommended dosage. All MLF treatments (including control fermentations) received a nutrient supplement, OptimaloPlus (Lallemand), at a dosage of 20 g/hL, on the second day after inoculation for MLF. This provides additional nutrients required by the bacteria for survival and completion of the fermentation.

Table 4.2 The characterised strains selected for evaluation in Pinotage in 2008 (individual strain fermentations). These strains were selected based on the results of the characterisation process.

***O. oeni* strains**	***Lb. plantarum* strains**
O. oeni A2	*Lb. plantarum* 14.1
O. oeni B1	*Lb. plantarum* 56
O. oeni S5	*Lb. plantarum* 66.1
O. oeni S6	*Lb. plantarum* 68
O. oeni E53	*Lb. plantarum* 71.1
O. oeni J65	*Lb. plantarum* 78.1
	Lb. plantarum 107

Evaluation of the individual LAB isolates took place in 2 L glass containers and was done in duplicate in Pinotage 2008. Evaluation of the mixed LAB treatments, consisting of an *O. oeni* and a *Lb. plantarum* strain, was performed in triplicate in 4.5 L glass containers in both vintages.

Table 4.3 Description of the MLF treatments performed in triplicate in 2008 with Pinotage, Cabernet Sauvignon and Shiraz. All treatments were inoculated with *S. cerevisiae* WE372 for AF.

Treatment number	**Mixed LAB cultures**		
1	*O. oeni* S5	and	*Lb. plantarum* 14.1
2	*O. oeni* S6	and	*Lb. plantarum* 14.1
3	*O. oeni* E53	and	*Lb. plantarum* 14.1
4	*O. oeni* S5	and	*Lb. plantarum* 56
5	*O. oeni* S6	and	*Lb. plantarum* 56
6	*O. oeni* E53	and	*Lb. plantarum* 56
7	*O. oeni* S5	and	*Lb. plantarum* 107
8	*O. oeni* S6	and	*Lb. plantarum* 107
9	*O. oeni* E53	and	*Lb. plantarum* 107
10	Lalvin VP41 (Lallemand)		
11	Spontaneous fermentation receiving no inoculation		

Table 4.4 Description of the treatment numbers and MLF treatments performed in triplicate in 2009 with Pinotage, Cabernet Sauvignon and Chardonnay. All red wine treatments were inoculated with *S. cerevisiae* WE372 for AF and Chardonnay was inoculated with *S. cerevisiae* VIN2000 for AF.

Description	Pinotage (seq.) *	Pinotage (co.) **	Cabernet Sauvignon (co.)	Chardonnay (co.)
Spontaneous fermentation	1 ***	1	1	
O. oeni S5 + *Lb. plantarum* 56	2	2	2	1
O. oeni S6 + *Lb. plantarum* 107	3	3	3	2
O. oeni E53 + *Lb. plantarum* 14.1	4	4	4	3
Lalvin VP41 (Lallemand)	5	5	5	4
ML01		6	6	5

* sequential inoculation
** co-inoculation
*** treatment number

The enumeration of the LAB strains prior to inoculation in the wine were as follow: *Lb. plantarum* and *O. oeni*, were grown at 30°C on De Man, Rogosa and Sharpe (MRS) agar and MRS agar plates supplemented with 10% tomato juice (All Gold, South Africa) (MRST) respectively, until single colonies were clearly distinguishable. All LAB were anaerobically cultivated by using Microbiology Anaerocult sheets in anaerobic jars (Merck, Darmstadt, Germany). MRS plates contained 50 g/L MRS broth (Biolab, Merck) and 15 g/L Bacteriological agar (Biolab, Merck). MRST plates contained 50 g/L MRS, 20 g/L Bacteriological agar supplemented with 10% preservative free tomato juice and with pH adjusted to 5.0 with hydrochloric acid (HCl). All plates contained 50 mg/L Delvocid Instant (DSM Food Specialties, The Netherlands) to prevent the growth of yeasts and 25 mg/L Kanamycin sulphate (Roche Diagnostics GmbH, Mannheim, Germany) to suppress the growth of acetic acid bacteria. MRS and MRST agar plates were incubated at 30°C for four and seven days, respectively. After growth on the agar plates, *Lb. plantarum* strains were grown at 30°C in MRS broth for two days. *Oenococcus oeni* strains were grown at 30°C for four days in filter-sterilised broth containing 50 g/L MRS broth supplemented with 20% preservative free apple juice (Ceres, South Africa) (MRSA) and with the pH adjusted to 5.2 with HCl.

Prior to inoculation in the wine, selected LAB strains were enumerated in an adaptation medium (**Table 4.5**) to ensure survival in the wine after inoculation. LAB strains grown in MRS and MRSA broth were inoculated in the adaptation media at 1.5 % and incubated at 30°C for two days. The adaptation media for *O. oeni* strains were filter-sterilised using a 0.22 µm syringe filter (Lasec) and also supplemented with 20 % preservative free apple juice. After two days, individual LAB strains were inoculated at approximately 10^6 cfu/mL in the wine, which resulted in a total population of $2x10^6$ cfu/mL in the mixed fermentations.

Table 4.5 The adaptation medium used to enumerate LAB strains prior to inoculation in the wine.

	***Lb. plantarum* (Autoclaved)**	***O. oeni* (Filter sterilised)**
MRS (Biolab, Merck)	50 g/L	50 g/L
(D-)-fructose (Merck)	40 g/L	40 g/L
(D+)-glucose (Merck)	20 g/L	20 g/L
(L-)-malic acid (Sigma)	4 g/L	4 g/L
Tween 80 (Merck)	1 g/L	1 g/L
Ethanol (absolute)	4%	4%
Apple juice (preservative free)	-	20%
pH	4.6	4.6

MLF in the wine was monitored by obtaining the concentrations of malic acid and lactic acid on a regular basis (start of MLF and every seven days) until its completion (taken as point when the malic acid concentration was equal to or lower than 0.3 g/L). This was done by Fourier-transform infrared spectroscopy (FT-IR) (WineScan FT120, FOSS Analytical, Denmark) as well as determination of the malic acid concentration with an enzymatic kit (Roche, Boehringer Mannheim, Germany). At these monitoring intervals, spectra were simultaneously acquired by FT-IR spectroscopy (**Section 4.2.3**).

After the completion of MLF, the wines were removed from the yeast lees and 50 to 80 mg/L SO_2 was added to all wines prior to bottling to achieve a total SO_2 concentration of 80 ppm.

4.2.2 MICROBIOLOGICAL ANALYSIS

Representative samples of the grape must were drawn for microbial enumeration, before MLF and on a weekly basis during MLF for both the 2008 and 2009 vintage.

The wine microbiological status was monitored by plate counts of colonies formed (colony forming units per millilitre, cfu/mL) on various agar media. A volume of 100 µL of grape must or wine, diluted in sterile, de-ionised water in a ten-fold dilution series, was plated. The plates were incubated at 30°C for four to seven days depending on the growth of the microorganisms. The colonies were counted and the cfu/mL determined and colonies were inspected by light microscopy to investigate the cell morphology. For the enumeration of *Lb. plantarum* and *O. oeni*, strains were grown as described previously.

ML01 (Warrenchem), the malolactic yeast, was enumerated on Yeast Peptone Dextrose (YPD) agar (Biolab, Merck) plates. All plates contained 25 mg/L Kanamycin sulphate (dissolved in 1 mL of sterile dH_2O) to suppress the growth of acetic acid bacteria and 30 mg/L Chloramphenicol (Roche Diagnostics GmbH, Mannheim, Germany) (dissolved in 1 mL of 96% ethanol) to suppress the growth of LAB. Plates were incubated aerobically at 30°C for two days.

4.2.3 STANDARD WINE ANALYSIS

Standard wine analysis of the juice and wine were performed with a WineScan FT120 spectrophotometer (FOSS Analytical, Denmark). The instrument utilises FT-IR. All samples were degassed by successive filtrations using a Filtration Unit (type 70500, FOSS Electric, Denmark) with 185 mm diameter filter paper sheets graded at 20 to 25 µm (Scheicher & Schuell, catalogue no. 10312714) connected to a vacuum pump. This effectively reduced the carbon dioxide (CO_2) levels to below 30 mg/L, as indicated by the FOSS WineScan. These concentrations are low enough not to interfere with the generation of the spectral data. Duplicate scans were obtained for each sample.

The standard analysis of the juice include: pH, volatile acid and total acid concentration, reducing sugars, malic acid concentration, glucose and fructose concentrations and density. The standard analysis of the wine include: pH, volatile and total acidity, malic and lactic acid, glucose and fructose, ethanol and glycerol. SO_2 (total and free) analysis was carried out using the Metrohm titration unit (Metrohm Ltd., Switzerland).

The primary aim of these scans was for routine monitoring of wine chemical compounds, but was also used for the generation of spectra in the wavenumber region 929-5011 cm^{-1} used for data analysis (**Section 4.2.5**). Spectral acquisition and processing took place as follow: degassed wine samples (7 mL) were pumped through the Ca_2F-cuvette (37 µm) of the spectrometer at a constant temperature of 40°C. The samples were scanned at 4 cm^{-1} intervals in the wavenumber region 929-5011 cm^{-1}. The amount of infrared radiation transmitted by the sample at all the infrared wavenumbers was captured simultaneously at the detector in the form of an interferogram which is converted with the Fourier Transform algorithm to a single beam transmittance spectrum (WineScan FT120 Type 771100 and 77310 Reference Manual, FOSS Analytical, Denmark, 2001).

Although the whole spectral range (929-5011 cm^{-1}) was stored for each sample, the wavenumbers 964-1532 cm^{-1} and 1716-2731 cm^{-1} were selected to exclude spectral noise largely caused by the absorption of water (Nieuwoudt *et al*., 2004) in the spectra used for multivariate data analysis.

4.2.4 DETERMINATION OF VOLATILE AROMA COMPOUNDS

Two methods were implemented in the determination of the volatile aroma compounds present in the finished wines. The major volatile aroma compounds were determined using gas chromatography with flame ionisation detection (GC-FID) and the carbonyl compounds were quantified using gas chromatography-mass spectrometry (GC-MS).

4.2.4.1 GC-FID chemicals, extraction method and conditions

Samples were collected from the bottled wines produced with the mixed LAB starter cultures from the 2008 and 2009 vintages. Volatile fatty acids, esters and alcohols (**Table 4.6**) were quantified in wine samples by GC-FID. Prior to analysis the volatile compounds were extracted from the wine

samples (5 mL wine sample) with 100 µL of internal standard followed by 1 mL of diethyl ether (Merck). The internal standard constituted 0.5 mg/L 4-methyl-2-pentanol (Fluka) in a model wine solution (12 % v/v ethanol, Merck; 2.5 g/L tartaric acid, Merck; de-ionised water from a MilliQ system; pH adjusted to 3.5 with 0.1 M NaOH, Merck). The wine/ether mixture was sonicated for 5 minutes followed by centrifugation at 4000 rpm for 3 minutes. The ether layer was removed and dried on sodium sulphate (Merck) (Louw, 2007). Each extract was injected into the GC-FID in triplicate. All chemicals used were analytical grade.

The gas chromatograph used for analysis (Hewlitt Packard 6890 Plus GC) was equipped with a split/splitless injector and a flame ionisation detector (Agilent, Little Falls, Wilmington, USA). Compounds were separated on a J & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m length x 0.32 mm internal diameter x 0.5 µm film thickness. The initial oven temperature was 33°C held for 17 minutes after which the temperature was increased to 240°C at 12°C/min and held for 5 minutes. The injection volume was 3 µL, at an injector temperature of 200°C. The split ratio was 15:1 and the split flow rate 49.5 mL/min. The column flow rate was 3.3 mL/min and the total run time 50 minutes per sample. The detector temperature was 250°C. After each sample run, a post run of 5 minutes at oven temperature 240°C was performed with a gas flow of 6 mL/min to clean the column. After every 30 samples the column was thermally and chemically cleaned by injecting hexane at oven temperature 220°C and holding it for 10 minutes (Louw, 2007). Volatile compound peak integration of the chromatograms was performed using HP Chemstation software (Rev. B01.03 [204]).

Table 4.6 The 26 volatile aroma compounds quantified by GC-FID analysis in the 2008 and 2009 mixed MLF treatments.

Volatile alcohols	Volatile Acids	Volatile Esters
Methanol	Acetic Acid	Ethyl Acetate
Propanol	Propionic Acid	Ethyl Butyrate
Isobutanol	Iso-butyric Acid	Isoamyl Acetate
Butanol	Butyric Acid	Ethyl Hexanoate
Isoamyl Alcohol	Iso-valeric Acid	Hexyl Acetate
Hexanol	Valeric Acid	Ethyl Lactate
2-Phenylethanol	Hexanoic Acid	Ethyl Caprylate
	Octanoic Acid	Ethyl Caprate
	Decanoic Acid	Diethyl Succinate
		2-Phenyl Acetate

4.2.4.2 GC-MS chemicals, extraction method and conditions

A method for the extraction and quantification of major wine carbonyl compounds was developed based on the method described by Hayasaka & Bartowsky (1999). Samples were collected from the bottled wines produced with the MLF treatments in the 2009 vintage. The carbonyl compounds diacetyl, acetoin and 2,3-pentanedione in wine samples were quantified by using Solid-phase microextraction (SPME) combined with GC-MS. Prior to analysis the carbonyl compounds were extracted from the wine samples. In a head space sampling vial 2 g of sodium chloride (Saarchem, Merck) was weighed out. One millilitre of wine sample and 9 mL of milliQ water (Millipore water purification system) was added, followed by the addition of 100 µL of internal standard, 2-pentanone (Fluka). The internal standard constituted 10 ppm 2-pentanone (Fluka) in a model wine solution (12 % v/v ethanol, Merck; 2.5 g/L tartaric acid, Merck; de-ionised water from a MilliQ system; pH adjusted to 3.5 with 0.1 M NaOH, Merck). The vials were capped and vortexed until the salt had been dissolved. Single injections of each sample were injected into the GC-MS. All chemicals used were analytical grade.

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 minutes) 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 x 250 µm internal diameter x 0.25 µm film thickness. The initial oven temperature was 35°C held for 5 minutes after which the temperature was increased to 150°C at 5°C/min and held for 2 minutes. The temperature was further increased to 240°C at 15°C/min and held for 1 minute. The split ratio was 10:1. The carrier gas was helium and the column flow rate was 1.7 mL/min.

4.2.5 DATA ANALYSIS

Multivariate data analysis techniques were used for statistical analysis. Principal component analyses (PCA) were performed in *The Unscrambler 9.2* Software (CAMO Process AS, Oslo, Norway) in order to observe underlying trends in the data.

4.2.6 INFORMAL SENSORIAL EVALUATION

The wines from the 2008 vintage that was made with the mixed isolates, were sensorially evaluated by seven wine panellists (lecturers, post-graduate students and staff from the Department of Viticulture and Oenology and the Institute for Wine Biotechnology and two industry affiliates) to determine the preferred combinations of LAB strains, as well as to select the combinations to be evaluated the following vintage. All wines were evaluated for aroma and taste

attributes and were presented at room temperature of 20°C. The wines were presented in clean, dry ISO wine glasses and covered with petri dishes in order to retain their aroma. Water and biscuits were used as palate cleansers in between wine tastings. Each taster was provided with a tasting sheet that listed the three cultivars and the treatments and was asked to generate descriptors and indicate preference.

4.3 RESULTS AND DISCUSSION

4.3.1 SMALL-SCALE VINIFICATION PROCEDURES

4.3.1.1 Alcoholic fermentation procedures

Grapes from the Stellenbosch and Wellington areas were used to evaluate the LAB isolates under winemaking conditions. The chemical composition of the grapes used in both the 2008 and 2009 vintages for co-inoculation, are listed in **Table 4.7**.

Table 4.8 shows the standard wine parameters after AF for varietals used for sequential inoculation in the two vintages. Initial malic acid concentrations varied, with the highest concentration present in the 2009 Chardonnay (3.71 g/L) and the lowest concentration found in the 2008 Shiraz (1.46 g/L). Alcohol concentrations for the different wines used for sequential inoculation (excluding Pinotage 2008) varied between 11.7 and 14.8 % v/v. The 2008 Pinotage was harvested at a very high sugar level which resulted in a very high alcohol concentration.

Table 4.7 Chemical composition of grape musts used for the vinification procedures during the 2008 and 2009 vintages.

Grape variety	Growing area	Harvest season	Sugar conc.*	Malic acid**	Total acidity**	pH
Pinotage	Wellington	2008	30.9	1.79	5.72	3.97
Pinotage	Wellington	2009	22.0	3.20	7.37	3.18
Cabernet Sauvignon	Stellenbosch	2008	23.4	3.35	7.77	3.63
Cabernet Sauvignon	Wellington	2009	23.1	1.66	8.29	3.46
Shiraz	Stellenbosch	2008	23.0	1.46	4.19	3.87
Chardonnay	Wellington	2009	20.2	3.71	8.30	3.28

* concentration in °B
** concentration in g/L

Table 4.8 Standard wine parameters of the different wines before inoculation with different MLF treatments. Red wine AF was conducted with *S. cerevisiae* WE372 and Chardonnay was inoculated with *S. cerevisiae* VIN2000.

Grape variety	Vintage	pH	Volatile acidity*	Total acidity**	Malic acid**	Lactic acid**	Ethanol***
Pinotage	2008	3.89	0.45	5.28	1.76	0.00	15.82
Cabernet Sauvignon	2008	3.67	0.12	6.89	3.36	0.05	12.78
Shiraz	2008	3.76	0.46	5.15	1.52	0.00	14.76
Pinotage	2009	3.57	0.31	6.91	2.81	0.05	11.78
Cabernet Sauvignon	2009	3.46	0.14	7.36	1.63	0.02	13.92
Chardonnay	2009	3.76	0.41	6.10	3.54	0.00	12.23

* concentration in g/L acetic acid
** concentration in g/L
*** concentration in v/v %

4.3.1.2 Malolactic fermentation procedures

4.3.1.2.1 Malolactic fermentation with individual isolates

4.3.1.2.1.1 Pinotage 2008

The individual isolates that were most successful in their fermentation performance in the synthetic wine medium, were further evaluated in Pinotage wine in 2008. Refer to **Table 4.8** for the standard wine parameters of Pinotage 2008 after AF and before inoculation with the different MLF treatments.

Of the seven *Lb. plantarum* strains that were evaluated, only three completed MLF (**Figure 4.1**) (malic acid concentration at or below 0.3 g/L). These were *Lb. plantarum* strains 14.1, 56, 107. The control Lalvin VP41 (Lallemand) showed the best performance and completed the fermentation in 23 days. Of the best performing *Lb. plantarum* strains, *Lb. plantarum* 107 finished fermentation in 30 days, compared to strains 56 and 14.1 which required 44 days The faster fermentation rate was evident despite the fact that *Lb. plantarum* 107 cell counts showed the most drastic decrease over the course of the fermentation (**Table 4.9**). The spontaneous fermentation and the remaining four strains never completed MLF. Microbiological analysis (**Table 4.9**) showed that all the treatments were inoculated at approximately $2x10^6$ to $4x10^6$ cfu/mL. *Lactobacillus plantarum* cell counts stayed constant or showed a slight decrease over the initial period of fermentation, followed by a steady decline towards the end of the fermentation. Cell counts of VP41 stayed constant over the course of the fermentation, whilst the indigenous microflora in the spontaneous fermentation demonstrated a rapid decrease in population numbers and as a result never completed MLF. Cell numbers eventually decreased to approximately 10^4 cfu/mL at completion of MLF.

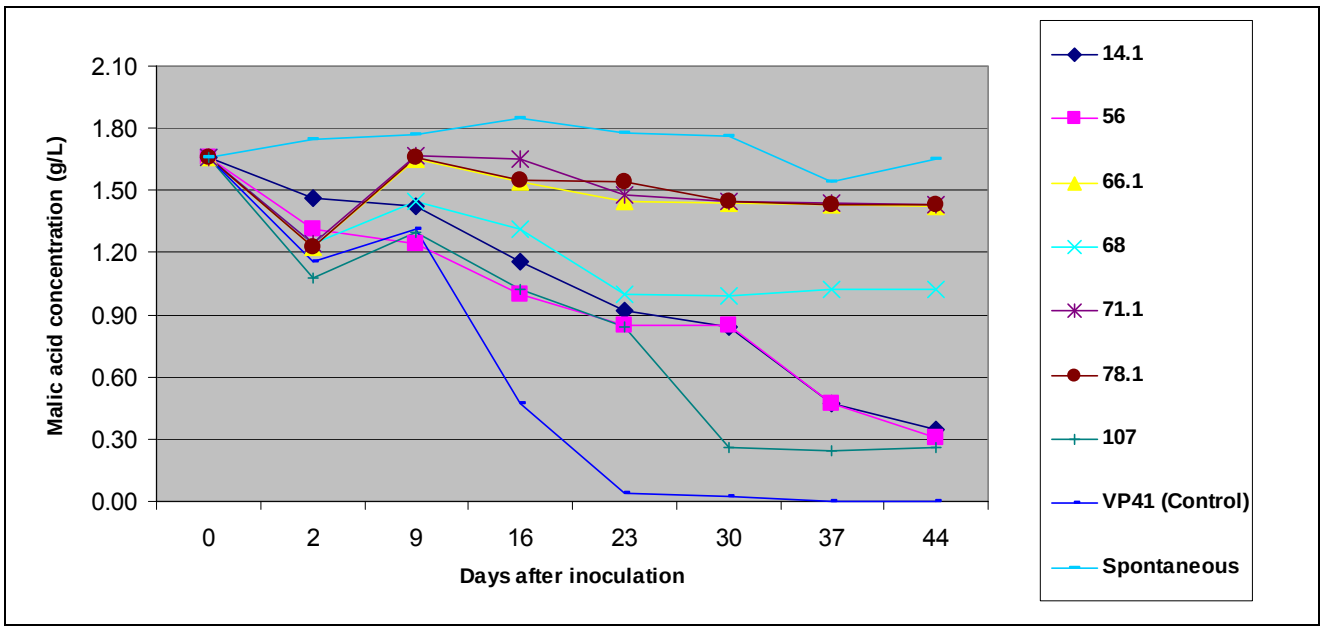

Figure 4.1 A fermentation graph showing the evolution of malic acid (g/L) in the MLF treatments evaluating the individual *Lb. plantarum* strains in Pinotage. Fermentations were monitored from the start of MLF on a weekly basis. Data shown indicate the average changes in the malic acid concentration (g/L) of each treatment repeated in duplicate. The relative standard deviation (RSD) is less than 10% between fermentation repeats.

Four of the eight *O. oeni* strains were able to complete MLF (**Figure 4.2**). Four of the isolates, *O. oeni* S5, S6, J65 and E53 completed MLF in nine days, showing a faster fermentation rate than VP41, which took 16 days to complete fermentation. The spontaneous treatment never completed MLF. Microbiological analysis showed that the *O. oeni* isolates were inoculated at approximately 10^7 cfu/mL (**Table 4.9**) and VP41 at $2x10^6$ cfu/mL. This could be a possible reason for the faster fermentation rate of the *O. oeni* isolates compared to VP41. Cell counts stayed constant during the early stages of MLF and slightly increased during the course of the fermentation. *Oenococcus oeni* B1 and J65 were the only treatments of which the population dropped below 10^7 cfu/mL and decreased to 10^6 cfu/mL at the end of fermentation.

Despite the fact that the *Lb. plantarum* strains took an average of 44 days to complete MLF, compared to nine days required by the *O. oeni* strains, this experiment served as an initial screening process to establish the ability of the isolates to survive in the challenging wine environment. This includes the ability to maintain sufficient viable cell numbers to successfully degrade malic acid and complete MLF. The LAB isolates also seem to be compatible with the yeast strain *S. cerevisiae* WE372 (Anchor). No immediate or drastic decrease in cell counts were observed after inoculation for MLF, which shows that metabolites and by-products produced by the wine yeast did not have a detrimental effect on the bacteria. Further investigation is required to elucidate the compatibility of the bacterial isolates with other wine yeast strains as well as the influence of different inoculation regimes on the yeast-bacteria interaction.

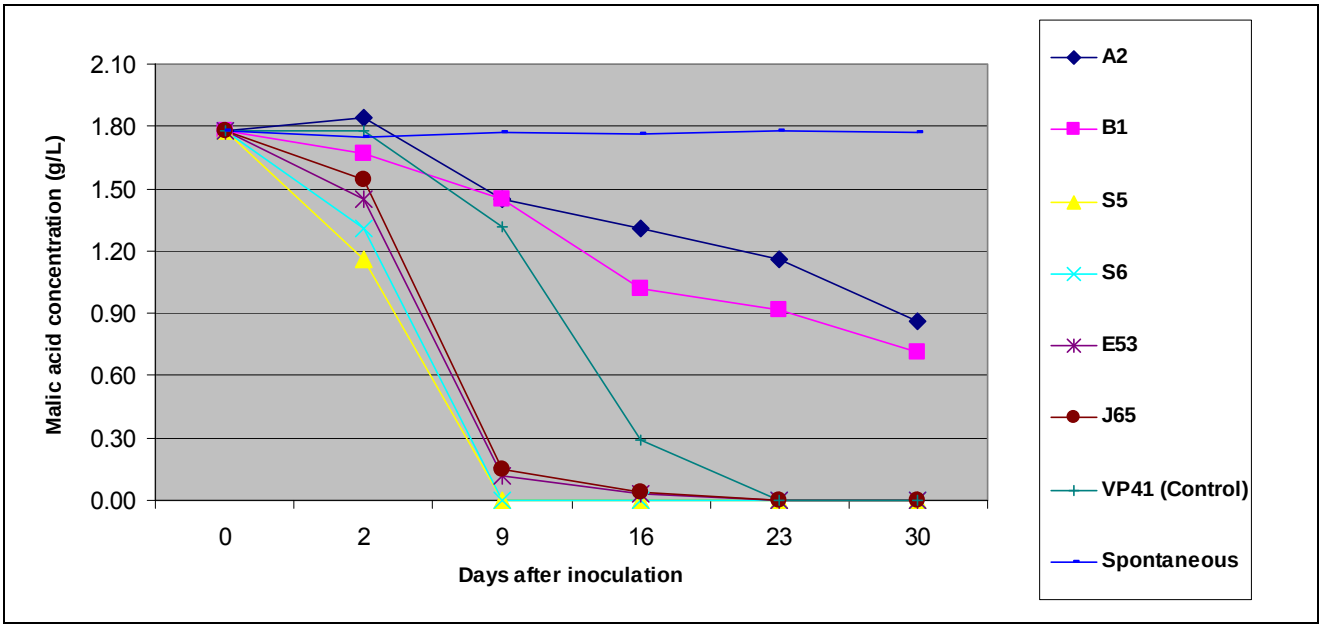

Figure 4.2 A fermentation graph showing the evolution of malic acid (g/L) in the MLF treatments evaluating the individual *O. oeni* strains in Pinotage. Fermentations were monitored from the start of MLF on a weekly basis. Data shown indicate the average changes in the malic acid concentration (g/L) of each treatment repeated in duplicate. The RSD is less than 10% between fermentation repeats.

Table 4.9 Cell counts (cfu/mL) of the individual LAB isolates and control fermentations as recorded at the start and different stages of MLF in Pinotage in 2008. Each enumeration represents the average of duplicate treatments (standard deviations not shown).

	Days after inoculation					
	0	2	9	16	23	30
Lb. plantarum 14.1	$2.9x10^6$	$1.2x10^6$	$1.3x10^6$	$1.0x10^6$	$3.2x10^5$	$1.0x10^5$
56	$3.1x10^6$	$3.0x10^6$	$1.3x10^6$	$5.0x10^5$	$1.3x10^5$	$8.4x10^4$
66.1	$3.6x10^6$	$3.9x10^6$	$7.9x10^5$	$2.4x10^5$	$7.1x10^4$	$1.0x10^4$
68	$3.0x10^6$	$2.7x10^6$	$4.8x10^5$	$9.0x10^4$	$1.4x10^4$	$1.0x10^4$
71.1	$3.5x10^6$	$1.7x10^6$	$2.9x10^5$	$1.8x10^5$	$7.9x10^4$	$4.0x10^4$
78.1	$4.9x10^6$	$2.4x10^6$	$2.9x10^5$	$1.5x10^5$	$7.6x10^4$	$2.4x10^4$
107	$3.3x10^6$	$2.7x10^6$	$1.0x10^6$	$3.5x10^4$	$2.7x10^3$	$1.2x10^3$
VP41	$3.2x10^6$	$1.3x10^6$	$1.0x10^6$	$1.2x10^6$	$2.8x10^6$	$4.3x10^6$
Spontaneous *	$2.0x10^6$	$3.0x10^4$	nd	nd	nd	nd

	Days after inoculation			
	0	2	9	16
O. oeni A2	$2.3x10^7$	$2.1x10^7$	$2.3x10^7$	$1.2x10^7$
B1	$2.5x10^7$	$1.5x10^7$	$1.5x10^7$	$8.9x10^6$
S5	$1.9x10^7$	$1.4x10^7$	$2.5x10^7$	$2.3x10^7$
S6	$1.7x10^7$	$1.3x10^7$	$2.3x10^7$	$2.4x10^7$
E53	$1.4x10^7$	$1.2x10^7$	$1.3x10^7$	$1.5x10^7$
J65	$1.7x10^7$	$1.2x10^7$	$4.0x10^6$	$4.0x10^6$
VP41	$2.1x10^6$	$1.9x10^6$	$1.7x10^6$	$3.0x10^6$
Spontaneous *	nd	nd	nd	nd

nd: not detected
* Spontaneous fermentations did not undergo MLF

Volatile acidity (measured as g/L acetic acid) is a wine parameter generally regarded as an indicator of quality. High levels of volatile acidity could have a detrimental effect on the sensory characteristics of the wine and generally imparts a pungent, vinegar-like aroma if present above the sensory threshold value. None of the individual LAB isolates resulted in volatile acidity concentrations exceeding the sensory threshold value of 0.7 g/L (Jackson, 2000) (**Table 4.10**). The seven *Lb. plantarum* strains showed comparable increases in the volatile acidity concentration. VP41 produced the highest concentration of 0.55 g/L. As expected, the spontaneous fermentations that did not complete MLF only demonstrated slight increases in the volatile acidity concentrations. The control fermentation of VP41 for the *O. oeni* fermentations showed the smallest increase in the volatile acidity concentration (**Table 4.10**). The three *O. oeni* strains with the lowest volatile acidity production, *O. oeni* A2, B1 and J65, resulted in an average increase of 0.06 g/L, compared to the three higher producing strains, *O. oeni* S5, S6 and E53, which were responsible for an average increase of 0.15 g/L in the volatile acidity concentration.

Table 4.10 A summary of the changes in volatile acidity (reported as g/L acetic acid) as a result of the MLF treatments with the individual isolates in Pinotage in 2008. Each value represents the average of duplicate treatment repeats at the start and end of MLF.

	Volatile acidity concentration (g/L)				
MLF treatment	**Before MLF**	**End of MLF**	**MLF treatment**	**Before MLF**	**End of MLF**
Lb. plantarum 14.1	0.45	0.49	***O. oeni*** A2	0.42	0.47
56	0.45	0.50	B1	0.42	0.49
66.1	0.45	0.48	S5	0.42	0.57
68	0.45	0.49	S6	0.42	0.57
71.1	0.45	0.50	E53	0.42	0.56
78.1	0.45	0.48	J65	0.42	0.48
107	0.45	0.51	VP41	0.42	0.46
VP41	0.45	0.55	Spontaneous *	0.42	0.44
Spontaneous *	0.45	0.48			

* Spontaneous fermentations did not undergo MLF

Based on both the fermentation rates, microbiological analysis and volatile acidity production, three strains each of *O. oeni* and *Lb. plantarum* were chosen for further evaluation in the study. The strains selected for evaluation in the mixed culture fermentations were *O. oeni* S5, S6 and E53 as well as *Lb. plantarum* strains 14.1, 56 and 107. These strains were able to survive in the wine environment, successfully degrade malic acid, retained sufficient cell counts to ensure the completion of MLF and did not produce concentrations of volatile acidity exceeding the sensory threshold.

4.3.1.2.2 Malolactic fermentation with mixed isolates

Three strains of each of *Lb. plantarum* and *O. oeni* were selected for evaluation in the mixed culture fermentations. The nine combinations, comprising of an *O. oeni* strain combined with a *Lb. plantarum* strain, were evaluated in Pinotage, Cabernet Sauvignon and Shiraz in 2008. Based on the performance of the mixed cultures in 2008, three combinations were chosen for further evaluation in 2009 in Pinotage, Cabernet Sauvignon and Chardonnay. Refer to **Table 4.3** for a description of the treatments.

4.3.1.2.2.1 2008

All nine MLF treatments (1 to 9) displayed similar trends in the three cultivars. All the treatments, except the spontaneous fermentation (treatment 11) that received no inoculation and never underwent MLF, were able to successfully complete fermentation. The respective fermentation rates of the mixture of LAB isolates in the three cultivars are shown in **Figures 4.3** to **4.5**, with **Table 4.11** summarising the time needed to complete MLF for the respective combinations.

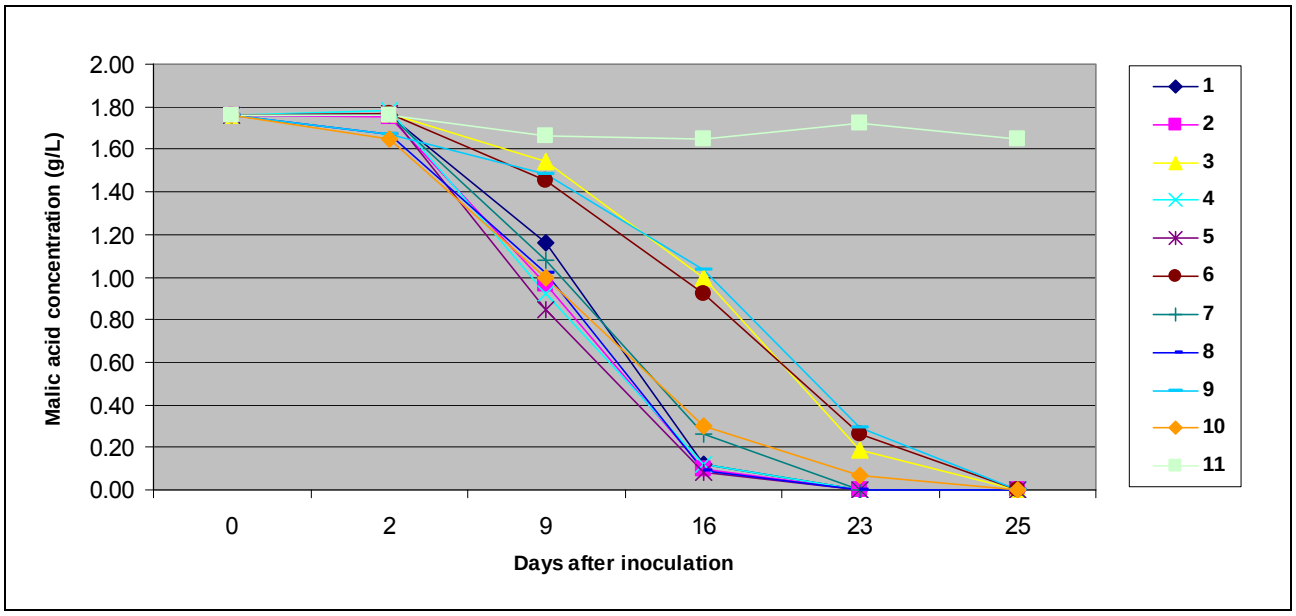

Figure 4.3 The fermentation graph of the mixed LAB treatments (refer to **Table 4.3** for treatment descriptions) during MLF in Pinotage in 2008. Each value represents the average of triplicate treatment repeats. The RSD is less than 10% between fermentation repeats.

In Pinotage, the treatments took 16 to 23 days to complete fermentation, 23 to 33 days in the Cabernet Sauvignon and 9 to 20 days in the Shiraz. Two groups can be distinguished based on their fermentation rates, which is interpreted as the rate at which malic acid is degraded by the bacterial population. The treatments containing *O. oeni* E53 (3, 6 and 9) took longer to complete MLF compared to the other treatments (1, 2, 4, 5, 7, 8 and 10), taking an extra 7, 10 and 11 days in the Pinotage, Cabernet Sauvignon and Shiraz, respectively, to complete fermentation. This trend was observed irrespective of the *Lb. plantarum* strain present in the mixture. This could imply that

the rate of fermentation is largely dependant on the fermentation tempo of the *O. oeni* strain present in the combination. The remaining treatments demonstrated fermentation rates comparable to each other and to that of the commercial control VP41, which completed MLF in 16, 23 and 9 days in Pinotage, Cabernet Sauvignon and Shiraz, respectively.

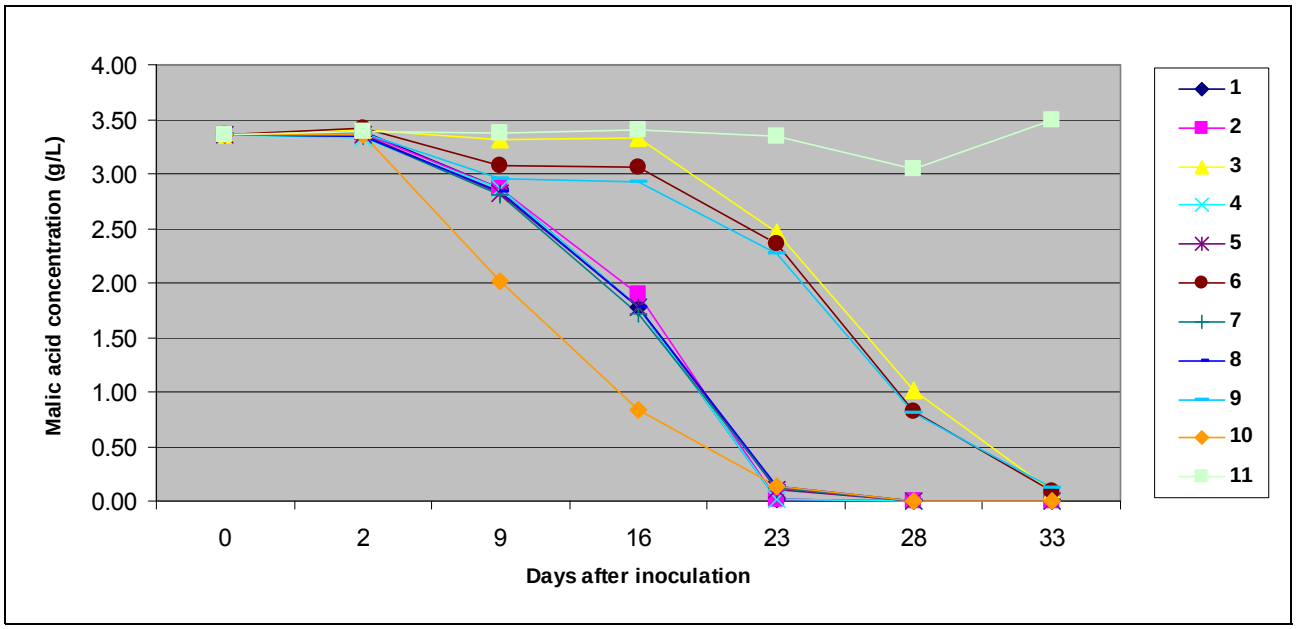

Figure 4.4 The fermentation graph of the mixed LAB treatments (refer to **Table 4.3** for treatment descriptions) during MLF in Cabernet Sauvignon in 2008. Each value represents the average of triplicate treatment repeats. The RSD is less than 10% between fermentation repeats.

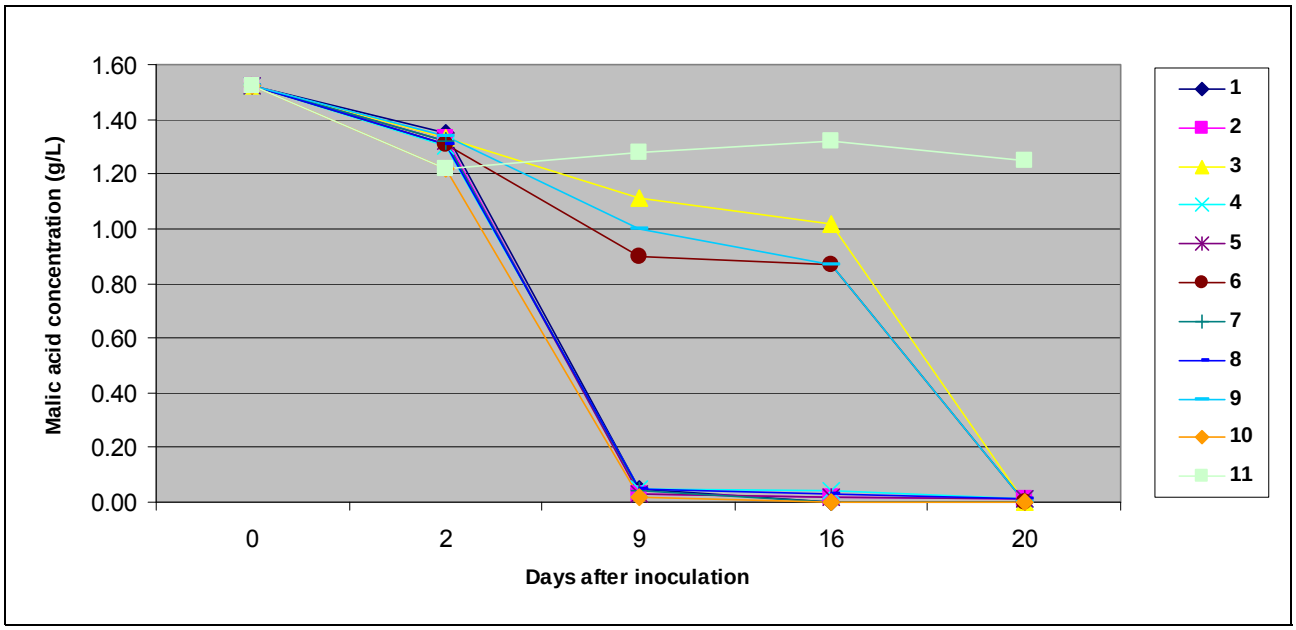

Figure 4.5 The fermentation graph of the mixed LAB treatments (refer to **Table 4.3** for treatment descriptions) during MLF in Shiraz in 2008. Each value represents the average of triplicate treatment repeats. The RSD is less than 10% between fermentation repeats.

The time to complete MLF also correlated with the initial concentration of malic acid present in the wine. In Cabernet Sauvignon, the treatments took the longest to complete fermentation and this cultivar also had the highest initial malic acid concentration (3.36 g/L), compared to the lower concentrations present in Pinotage and Shiraz. Based on their respective fermentation rates, there is very little to distinguish between the six treatments that were able to successfully complete MLF in the shortest time (1, 2, 4, 5, 7 and 8).

Table 4.11 A summary of the time needed by the mixed LAB treatments to complete MLF in Pinotage, Cabernet Sauvignon and Shiraz in 2008. Treatment 11 did not complete MLF. The duration is an average value determined in triplicate for each treatment.

	Time to complete MLF (days)		
Treatments *	**Pinotage**	**Cabernet Sauvignon**	**Shiraz**
1	16	23	9
2	16	23	9
3	23	33	20
4	16	23	9
5	16	23	9
6	23	33	20
7	16	23	9
8	16	23	9
9	23	33	20
10	16	23	9
11	-	-	-

* Refer to **Table 4.3** for a description of the treatments

There were no significant increases in the volatile acidity concentrations produced by any of the treatments and the final concentrations were below that of the sensory threshold of 0.7 g/L (Jackson, 2000) (data not shown). This was true for all three cultivars.

Microbiological analyses of the mixed fermentations were done during the course of the fermentations until MLF was completed. Cell counts (cfu/mL) were determined after four days for *Lb. plantarum* and after seven days for *O. oeni*. Neither of the media used for LAB enumeration were selective for either *O. oeni* or *Lb. plantarum*. It was however, in this study, possible to distinguish between *Lb. plantarum* and *O. oeni* colonies on the plates. *Lactobacillus plantarum* colonies grew at a faster rate and were visible after four days and were larger with a more yellow tint, compared to smaller, white *O. oeni* colonies which were only visible after seven days of incubation at 30°C. The results of the microbiological analysis of the mixed fermentations in 2008 are shown in **Tables 4.12** to **4.14**.

The Lalvin VP41 (Lallemand) control fermentations were inoculated at the recommended dosage of $1x10^6$ cfu/mL in all three cultivars. There were no cell counts detected in the spontaneous fermentations (treatment 11) except for the detection of *O. oeni* colonies in Shiraz on day 9 and 16.

In Pinotage (**Table 4.12**), the *Lb. plantarum* strains were inoculated at approximately

$7x10^5$ to $1x10^6$ cfu/mL. During the course of the fermentations, the cell counts of the *Lb. plantarum* strains decreased. *Lactobacillus plantarum* 107 (treatments 7, 8 and 9) decreased to an average of about $7x10^3$ cfu/mL, *Lb. plantarum* 14.1 (1, 2 and 3) decreased to approximately $8x10^4$ cfu/mL and *Lb. plantarum* 56 (4, 5 and 6) decreased to approximately $2x10^5$ cfu/mL. *Lactobacillus plantarum* 107 also showed the fastest decrease in cell numbers. *Oenococcus oeni* S5 and S6 (treatments 1, 4, 7, 2, 5 and 8) showed the best growth capability, compared to *O. oeni* E53 (3, 6 and 9).

In Cabernet Sauvignon (**Table 4.13**), *Lb. plantarum* strains were inoculated at cell counts of $6x10^5$ to $1x10^6$ cfu/mL. *Lactobacillus plantarum* cell counts decreased to between $1x10^4$ and $1x10^5$ cfu/mL towards the end of fermentation. Contrary to what was found in Pinotage, treatments containing *Lb. plantarum* 14.1 (1, 2 and 3) showed the fastest decrease in cell numbers, whilst the other treatments showed a steadier decline in cell numbers during the fermentation. *Oenococcus oeni* cell counts were determined as being between $1x10^6$ and $1x10^7$ cfu/mL. *Oenococcus oeni* E53 (treatments 3, 6 and 9) took the longest time to reach maximum cell numbers of 10^6 to 10^7 cfu/mL, which could be due to the possibility that this *O. oeni* strain demonstrates a longer lag phase, which could also be the reason that the treatments containing this strain took longer to complete MLF.

In Shiraz (**Table 4.14**), very few differences were evident between the treatments. All the *Lb. plantarum* strains were inoculated at $8x10^5$ to $1x10^6$ cfu/mL and decreased at a similar rate towards the end of the fermentation. Both *O. oeni* S5 (treatments 1, 4 and 7) and *O. oeni* S6 (2, 5 and 8) displayed similar trends in cell counts, but once again treatments containing *O. oeni* E53 (3, 6 and 9) took longer to reach 10^6 cfu/mL and adapt to the wine environment and therefore these fermentations took longer to complete. *Oenococcus oeni* S5 and S6 eventually reached $4x10^6$ to $6x10^6$ cfu/mL halfway through the fermentation, whilst the commercial control VP41 reached cell counts of up to $1.2x10^7$ cfu/mL. Despite the higher cell counts of VP41, fermentation rates of the treatments and the control were still of a comparable nature.

There is very little or no difference between the microbiological analysis of *O. oeni* S5 and S6 in the three cultivars, where only *O. oeni* E53 demonstrated a longer lag phase. The three *Lb. plantarum* strains only demonstrated small differences in their performance in the three cultivars.

Based on the performance of the different mixed LAB treatments in the 2008 vintage, combined with the sensorial data (to be discussed in **Section 4.3.4**), the three combinations that showed the most promise were selected for further evaluation in the 2009 vintage. Refer to **Table 4.4** for a description of the treatments that were selected for further use in this study.

Table 4.12 Cell counts (cfu/mL) of the mixed LAB cultures as recorded at the start and different stages of MLF in Pinotage in 2008. Each enumeration represents the average of triplicate treatments (standard deviations not shown).

	Days after inoculation									
	0		2		9		16		23	
Treatments *	***O. oeni***	***Lb.***	***O. oeni***	***Lb.***	***O. oeni***	***Lb.***	***O. oeni***	***Lb.***	***O. oeni***	***Lb.***
1	$3.0X10^5$	$6.5X10^5$	$3.0X10^5$	$6.3X10^5$	$7.6X10^5$	$2.6X10^5$	$5.3X10^6$	$7.4X10^4$	ndt	ndt
2	$4.5X10^5$	$7.3X10^5$	$6.0X10^5$	$6.9X10^5$	$1.2X10^6$	$2.2X10^5$	$3.7X10^6$	$9.0X10^4$	ndt	ndt
3	$3.0X10^5$	$7.0X10^5$	$3.0X10^5$	$6.0X10^5$	$4.6X10^5$	$2.3X10^5$	$4.0X10^6$	$8.4X10^4$	$7.5X10^6$	$1.6X10^4$
4	$2.0X10^5$	$7.7X10^5$	$3.0X10^5$	$7.2X10^5$	$5.9X10^5$	$3.0X10^5$	$4.2X10^6$	$1.5X10^5$	ndt	ndt
5	$1.5X10^5$	$1.0X10^6$	$3.0X10^5$	$6.7X10^5$	$1.3X10^6$	$3.4X10^5$	$4.7X10^6$	$1.6X10^5$	ndt	ndt
6	$3.0X10^5$	$8.9X10^5$	$2.0X10^5$	$5.9X10^5$	$3.7X10^5$	$3.5X10^5$	$3.6X10^6$	$1.6X10^5$	$8.5X10^6$	$4.4X10^4$
7	$2.5X10^5$	$1.1X10^6$	$2.0X10^5$	$7.7X10^5$	$1.6X10^6$	$1.2X10^5$	$6.4X10^6$	$7.5X10^3$	ndt	ndt
8	$2.5X10^5$	$8.5X10^5$	$4.0X10^5$	$8.1X10^5$	$1.8X100^6$	$1.1X10^5$	$4.7X10^6$	$7.0X10^3$	ndt	ndt
9	$2.0X10^5$	$8.5X10^5$	$1.5X10^5$	$8.1X10^5$	$2.8X10^5$	$1.1X10^5$	$3.7X10^6$	$7.0X10^3$	$1.0X10^7$	nd
10	$1.3X10^6$	nd	$1.2X10^6$	nd	$8.3X10^5$	nd	$1.9X10^6$	nd	$2.3X10^6$	nd
11	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

* Refer to **Table 4.3** for a description of the different treatments
** *Lb*: *Lactobacillus plantarum*
nd: not detected; ndt: not determined as MLF was completed

Table 4.13 Cell counts (cfu/mL) of the mixed LAB cultures as recorded at the start and different stages of MLF in Cabernet Sauvignon in 2008. Each enumeration represents the average of triplicate treatments (standard deviations not shown).

	Days after inoculation											
	0		2		9		16		23		33	
Treatments *	***O. oeni***	***Lb.*** **	***O. oeni***	***Lb.***	***O. oeni***	***Lb.***	***O. oeni***	***Lb.***	***O. oeni***	***Lb.***	***O. oeni***	***Lb.***
1	$5.50X10^5$	$6.90X10^5$	$2.00X10^5$	$3.00X10^5$	$7.10X10^5$	$1.40X10^5$	$9.40X10^6$	$4.00X10^4$	$1.32X10^7$	$1.10X10^4$	ndt	ndt
2	$5.00X10^5$	$8.00X10^5$	$1.10X10^5$	$2.50X10^5$	$9.20X10^5$	$1.30X10^5$	$8.00X10^6$	$5.10X10^4$	$1.00X10^7$	$1.30X10^4$	ndt	ndt
3	$2.00X10^5$	$7.80X10^5$	$1.00X10^4$	$2.00X10^5$	$1.40X10^4$	$1.40X10^5$	$2.10X10^5$	$2.30X10^4$	$3.60X10^6$	$3.50X10^4$	$2.10X10^7$	nd
4	$3.00X10^5$	$1.10X10^6$	$2.00X10^5$	$8.20X10^5$	$1.50X10^4$	$6.70X10^5$	$3.00X10^6$	$3.60X10^5$	$3.00X10^6$	$2.80X10^5$	ndt	ndt
5	$4.50X10^5$	$6.00X10^5$	$2.50X10^5$	$6.30X10^5$	$1.60X10^4$	$5.40X10^5$	$2.00X10^6$	$4.20X10^5$	$3.00X10^6$	$2.30X10^5$	ndt	ndt
6	$3.00X10^5$	$7.80X10^5$	$1.00X10^5$	$6.20X10^5$	$1.40X10^4$	$6.50X10^5$	$1.50X10^5$	$3.50X10^5$	$4.90X10^6$	$1.70X10^5$	$2.20X10^7$	$3.90X10^4$
7	$8.50X10^5$	$8.40X10^5$	$2.00X10^5$	$6.90X10^5$	$4.90X10^5$	$4.30X10^5$	$7.00X10^6$	$2.00X10^5$	$1.40X10^7$	$3.70X10^4$	ndt	ndt
8	$3.00X10^5$	$8.70X10^5$	$2.00X10^5$	$8.40X10^5$	$5.60X10^4$	$5.20X10^5$	$6.60X10^6$	$1.80X10^5$	$1.10X10^7$	$3.50X10^4$	ndt	ndt
9	$2.00X10^5$	$8.50X10^5$	$1.00X10^5$	$7.10X10^5$	$2.90X10^4$	$4.20X10^5$	$3.20X10^5$	$1.50X10^5$	$3.64X10^6$	$1.30X10^4$	$2.40X10^7$	nd
10	$1.40X10^6$	nd	$1.20X10^6$	nd	$3.50X10^6$	nd	$6.00X10^6$	nd	$4.50X10^6$	nd	ndt	ndt
11	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

* Refer to **Table 4.3** for a description of the different treatments
** *Lb*: *Lactobacillus plantarum*
nd: not detected; ndt: not determined as MLF was completed

Table 4.14 Cell counts (cfu/mL) of the mixed LAB cultures as recorded at the start and different stages of MLF in Shiraz in 2008. Each enumeration represents the average of triplicate treatments (standard deviations not shown).

	Days after inoculation							
	0		2		9		16	
Treatments *	***O. oeni***	***Lb. *****	***O. oeni***	***Lb.***	***O. oeni***	***Lb.***	***O. oeni***	***Lb.***
1	$1.5x10^5$	$1.2x10^6$	$1.0x10^5$	$7.6x10^5$	$5.9x10^6$	$1.8x10^5$	ndt	ndt
2	$6.0x10^5$	$9.0x10^5$	$4.5x10^5$	$6.9x10^5$	$4.6x10^6$	$2.0x10^5$	ndt	ndt
3	$3.0x10^5$	$8.0x10^5$	$1.0x10^4$	$6.8x10^5$	$3.0x10^4$	$1.5x10^5$	$2.5x10^6$	$1.4x10^4$
4	$4.0x10^5$	$9.4x10^5$	$4.0x10^5$	$7.0x10^5$	$6.0x10^6$	$1.7x10^5$	ndt	ndt
5	$1.0x10^6$	$8.5x10^5$	$3.0x10^5$	$5.4x10^5$	$4.2x10^6$	$1.8x10^5$	ndt	ndt
6	$1.0x10^5$	$1.1x10^6$	$1.0x10^5$	$8.3x10^5$	$3.7x10^4$	$1.2x10^5$	$1.9x10^6$	$1.5x10^4$
7	$7.0x10^5$	$8.6x10^5$	$1.0x10^5$	$6.4x10^5$	$5.0x10^6$	$9.0x10^4$	ndt	ndt
8	$3.5x10^5$	$7.6x10^5$	$5.0x10^5$	$7.0x10^5$	$5.0x10^6$	$1.4x10^5$	ndt	ndt
9	$1.0x10^5$	$7.6x10^5$	$6.0x10^4$	$8.0x10^5$	$4.6x10^4$	$6.8x10^4$	$2.8x10^6$	$2.0x10^3$
10	$2.0x10^6$	nd	$1.2x10^6$	nd	$1.2x10^7$	nd	ndt	ndt
11	nd	nd	nd	nd	$1.3x10^2$	nd	$1.3x10^3$	nd

* Refer to **Table 4.3** for a description of the different treatments
** *Lb*: *Lactobacillus plantarum*
nd: not detected; ndt: not determined as MLF was complete

4.3.1.2.2.2 2009

A) Pinotage

The treatments were evaluated by inoculating the LAB after the completion of AF (sequential inoculation) and also in a co-inoculation scenario, where LAB were inoculated 24 hours after the yeast (*S. cerevisiae* WE372 from Anchor Yeast) had been inoculated for AF. The ML01 yeast completed AF in the same time frame as the other treatments, unless stated otherwise. Refer to **Table 4.4** for a description of the treatments evaluated in Pinotage in 2009.

The evolution of malic acid during the fermentations with the mixed LAB treatments and the two inoculation times, are shown in **Figures 4.6** and **4.7**. All three the co-inoculated mixed culture treatments (2, 3, and 4) completed MLF in nine days, the same amount of time as the commercial culture VP41 (treatment 5) and 14 days faster than the ML01 malolactic wine yeast (treatment 6). The ML01 yeast initiated and started MLF at the fastest rate, but eventually took the same amount of time as the spontaneous control (treatment 1) to complete MLF.

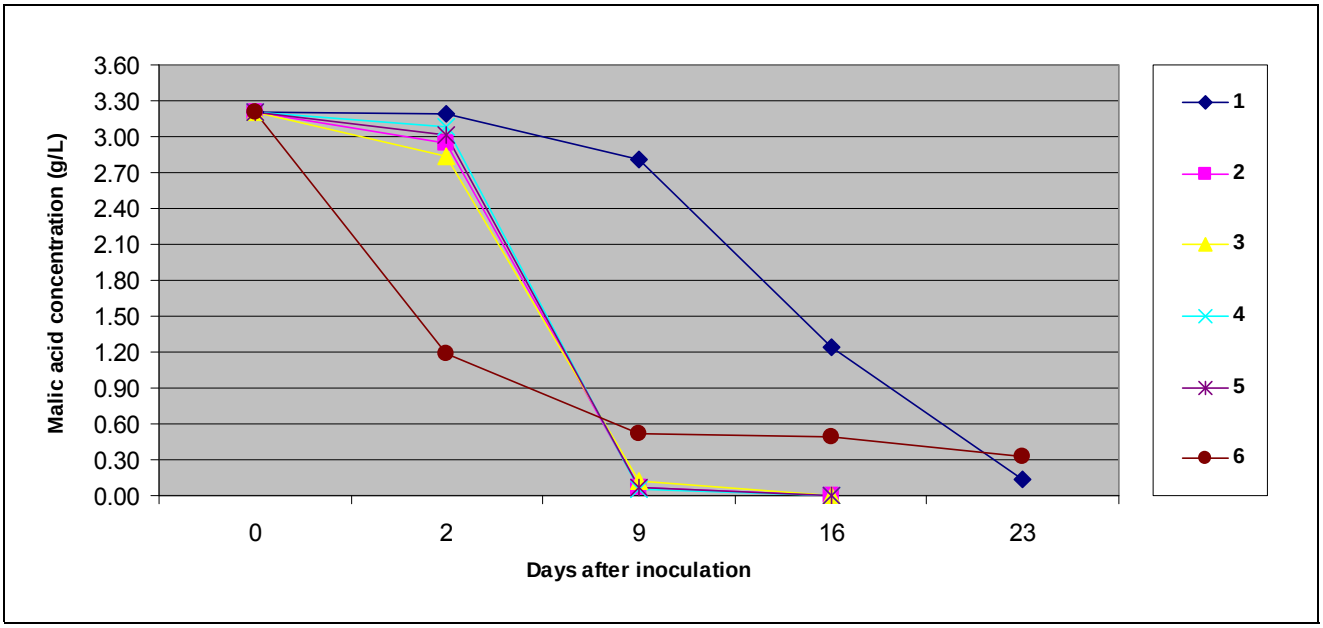

Figure 4.6 A fermentation graph showing the evolution of malic acid (g/L) in the MLF treatments evaluating the mixed strain combinations in Pinotage 2009 in a co-inoculation scenario (refer to **Table 4.4** for treatment descriptions). *Saccharomyces cerevisiae* WE372 was used as the commercial culture for AF inoculation. Fermentations were monitored from the start of fermentation on a weekly basis until completion. Data shown indicate the average changes in the malic acid concentration of each treatment repeated in triplicate. The RSD is less than 10% between fermentation repeats.

Similar results were observed in the sequential inoculation treatments. The MLF treatments took 9 to 16 days to complete fermentation and treatments 2 and 3, the first two mixed culture treatments, finished MLF in nine days, compared to combination three (treatment 4) and the commercial control VP41 (treatment 5) that finished MLF after an additional seven days. The

spontaneous fermentation (treatment 1) also completed MLF and took 30 days until completion. It is therefore possible that the spontaneous LAB could have contributed to the MLF in the mixed culture treatments. It is clear from **Figure 4.7** that the three combinations of mixed LAB (treatments 2, 3 and 4) had very similar fermentation rates and either completed MLF in the same time frame or faster than the commercial control.

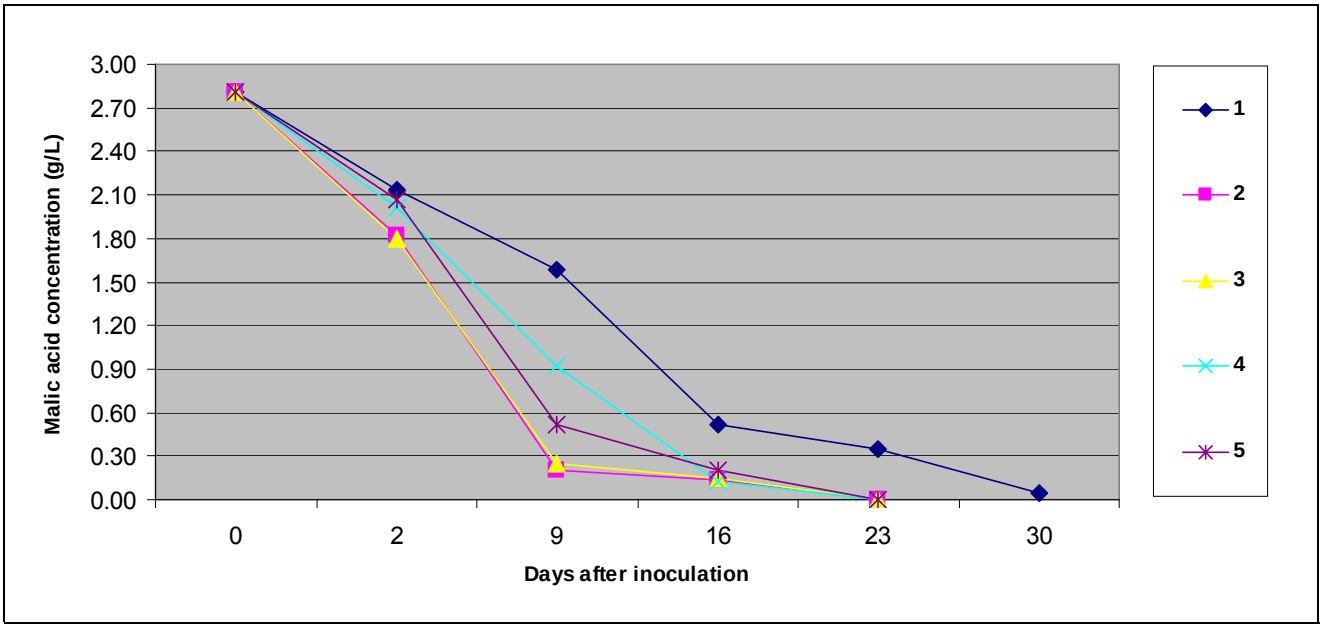

Figure 4.7 A fermentation graph showing the evolution of malic acid (g/L) in the MLF treatments evaluating the mixed strain combinations in Pinotage 2009 in a sequential inoculation scenario (refer to **Table 4.4** for treatment descriptions). *Saccharomyces cerevisiae* WE372 was used as the commercial culture for AF inoculation. Fermentations were monitored from the start of fermentation on a weekly basis until completion. Data shown indicate the average changes in the malic acid concentration of each treatment repeated in triplicate. The RSD is less than 10% between fermentation repeats.

Similar concentrations of volatile acidity were produced by the mixed cultures in the two inoculation scenarios (**Figures 4.8** and **4.9**). Average concentrations of 0.44 to 0.50 g/L were produced by the three combination treatments (2, 3 and 4) for both inoculation times. The co-inoculation spontaneous control and ML01 treatments (1 and 6) produced the lowest levels of volatile acid, whilst the VP41 treatment (5) produced the highest concentration of 0.54 g/L. Due to the fact that the spontaneous control (treatment 1) also underwent MLF, we cannot determine the amount of volatile acid production that can be attributed to AF and the yeast with certainty. For the sequential inoculation, the two control fermentations (1 and 5) produced the lowest concentrations of volatile acidity, with similar concentrations being produced by the three combination treatments (2, 3 and 4), concentrations of 0.45 g/L, 0.46 g/L and 0.44 g/L, respectively. Despite the fact that all the treatments resulted in increases in the volatile acidity concentration, these increases are potentially sensorial insignificant and below the sensory threshold value. The assumption that MLF in a co-inoculation scenario could produce much higher concentrations of volatile acid was not found in this study (Semon *et al*., 2001).

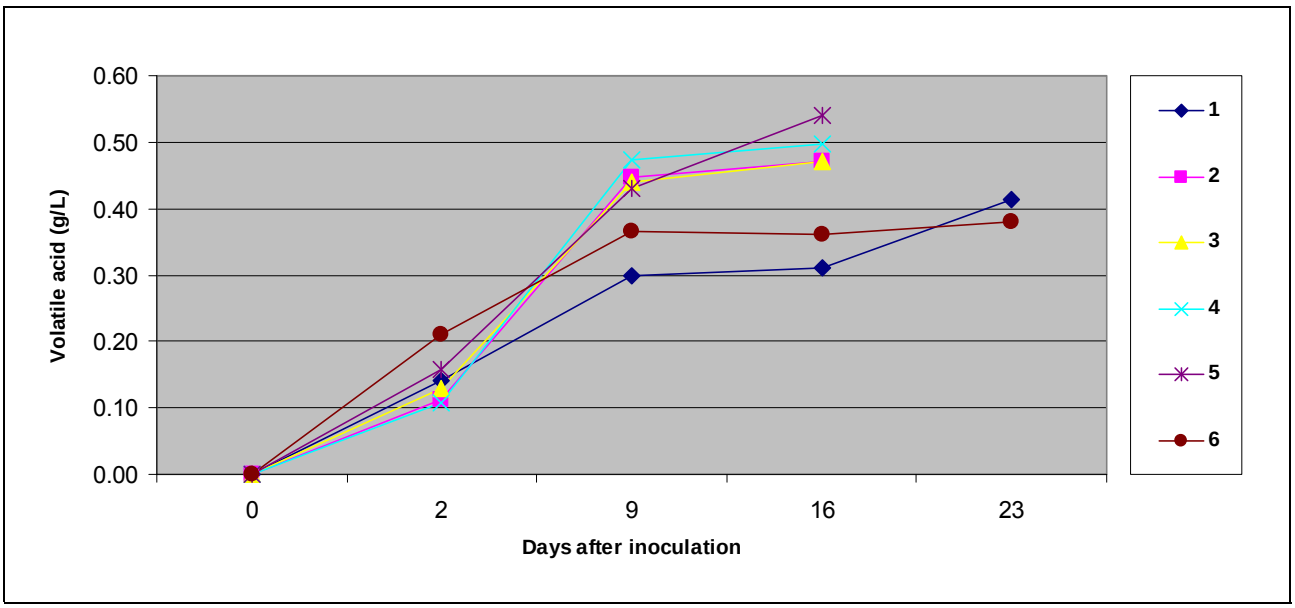

Figure 4.8 The increase in volatile acidity (reported as g/L acetic acid) of the different MLF treatments (refer to **Table 4.4** for treatment descriptions) in Pinotage 2009 under co-inoculation conditions. Each value represents the average of triplicate treatments. The RSD is less than 10% between fermentation repeats.

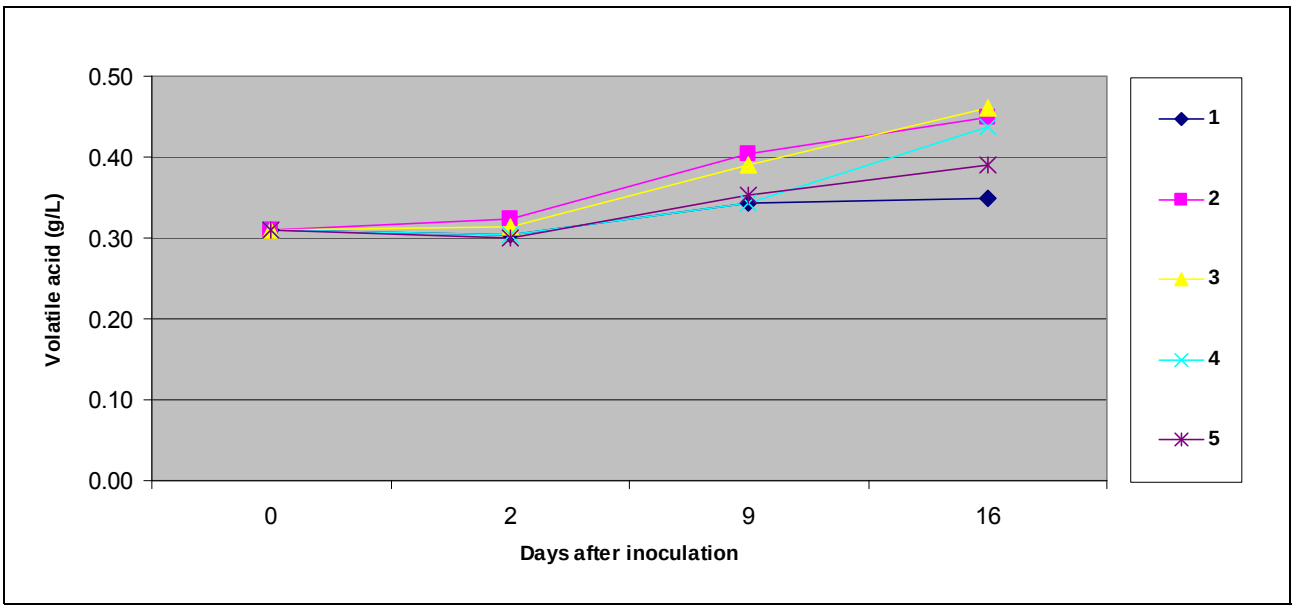

Figure 4.9 The increase in volatile acidity (reported as g/L acetic acid) of the different MLF treatments (refer to **Table 4.4** for treatment descriptions) in Pinotage 2009 in sequential inoculation conditions. Each value represents the average of triplicate treatments. The RSD is less than 10% between fermentation repeats.

Microbiological analysis of the co-inoculated wines (**Table 4.15**) indicated that all the treatments were inoculated at approximately 10^6 cfu/mL. All the treatments (2 to 5), the mixed cultures as well as VP41, showed increases in the microbial population, reaching cell counts of 10^7 cfu/mL. The spontaneous fermentation (treatment 1) saw a decrease in the indigenous LAB population at the start of AF, most likely due to the increase in ethanol and other yeast-derived

metabolites. Cell counts eventually increased as the bacteria adapted to the environment and spontaneous MLF took place. The ML01 yeast population (treatment 6) fluctuated between 10^6 and 10^7 cfu/mL during the course of the fermentation. It is clear that there were no adverse interactions between the yeast strain inoculated for AF and the mixed LAB cultures. Co-inoculation seems to be a feasible inoculation option for the mixed LAB starter cultures.

In results similar to that of the co-inoculated wines, all of the sequential inoculation treatments had initial population cell counts of 10^6 cfu/mL and increased to 10^7 cfu/L during the course of the fermentation (**Table 4.16**). The mixed LAB cultures successfully completed MLF in both the co-inoculation, as well as sequential inoculation scenarios.

Table 4.15 Cell counts (cfu/mL) of the total LAB population (treatments 1 to 5) and yeast population (treatment 6) of the mixed LAB fermentations as recorded at the start and different stages of MLF in Pinotage 2009 in co-inoculation with *S. cerevisiae* WE372 (treatments 1 to 5). Each enumeration represents the average of triplicate treatments (standard deviations not shown).

	Days after inoculation				
Treatment *	**0**	**2**	**9**	**16**	**23**
1	$1.2x10^6$	$2.0x10^3$	$3.0x10^4$	$4.2x10^6$	$3.2x10^7$
2	$3.8x10^6$	$1.3x10^6$	$7.6x10^7$	$1.3x10^7$	ndt
3	$3.0x10^6$	$2.1x10^6$	$8.5x10^7$	$1.6x10^7$	ndt
4	$4.3x10^6$	$8.0x10^5$	$6.8x10^7$	$4.6x10^7$	ndt
5	$6.5x10^6$	$1.6x10^6$	$4.5x10^7$	$4.2x10^7$	ndt
6	$6.9x10^6$	$8.1x10^6$	$1.0x10^7$	$4.3x10^6$	$2.1x10^6$

* Refer to **Table 4.4** for treatment descriptions
ndt: not determined as MLF was completed

Table 4.16 Cell counts (cfu/mL) of the total LAB population of the mixed LAB fermentations as recorded at the start and different stages of MLF in Pinotage 2009 in sequential inoculation with *S. cerevisiae* WE372. Each enumeration represents the average of triplicate treatments (standard deviations not shown).

	Days after inoculation				
Treatment *	**0**	**2**	**9**	**16**	**23**
1	$5.9x10^6$	$3.8x10^7$	$1.1x10^5$	$1.4x10^7$	$3.0x10^7$
2	$1.9x10^6$	$3.4x10^7$	$2.5x10^7$	$2.7x10^7$	ndt
3	$1.7x10^6$	$1.1x10^7$	$2.5x10^7$	$3.0x10^7$	ndt
4	$2.6x10^6$	$3.4x10^7$	$6.1x10^6$	$2.7x10^7$	ndt
5	$1.1x10^6$	$3.5x10^6$	$1.1x10^7$	$2.0x10^7$	ndt

* Refer to **Table 4.4** for treatment descriptions
ndt: not determined as MLF was completed

B) Cabernet Sauvignon

Inoculation of the mixed culture treatments in sequential inoculation was unsuccessful. Sulphur levels were within accepted limits and could not account for the difficulty in initiating MLF. Treatments were re-inoculated for MLF after removal of the yeast lees, but microbiological analysis of the wines showed that there were no bacteria present after inoculation. Treatment of the wine with Malostart (Laffort), a product that mainly consists of yeast hulls to remove potentially harmful metabolites like medium chain fatty acids produced by the yeast, followed by removal of the sediment and re-inoculation, was also unsuccessful. Therefore only co-inoculation results were obtained for Cabernet Sauvignon in the 2009 vintage.

The three treatments with the mixed LAB (2, 3 and 4) completed MLF in nine days (**Figure 4.10**), displayed fermentation rates that were comparable to that of VP41 (treatment 5), the commercial control, that also completed MLF in nine days. Contrary to the results observed in the Pinotage co-inoculation results, neither the spontaneous fermentation (treatment 1), nor the ML01 wine yeast (treatment 6) was able to complete MLF. It is possible that the indigenous microflora and the ML01 yeast might be more sensitive to the higher levels of phenols inherently present in Cabernet Sauvignon (Klenar *et al*., 2004; Ertan Anli and Vural, 2009).

Figure 4.11 depicts the increase in volatile acidity of the co-inoculation treatments. Only minimal increases in volatile acidity were observed for the co-inoculation treatments.

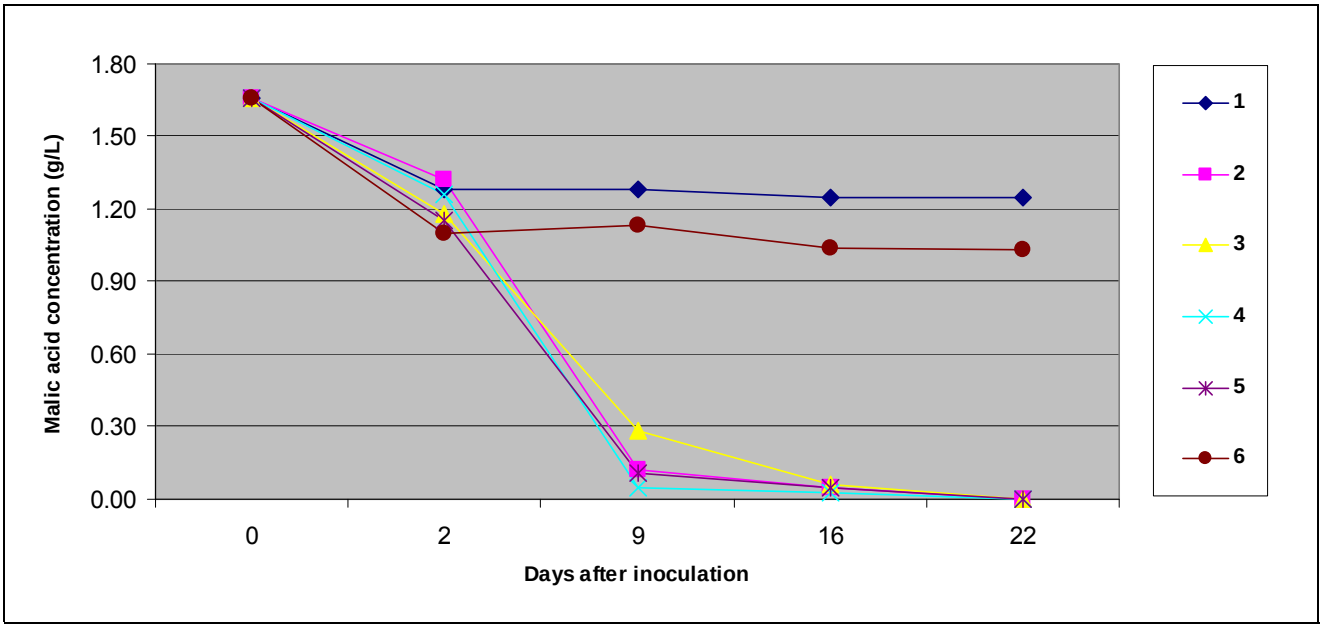

Figure 4.10 A fermentation graph showing the evolution of malic acid (g/L) in the MLF treatments evaluating the mixed strain combinations in Cabernet Sauvignon 2009 in a co-inoculation scenario (refer to **Table 4.4** for treatment descriptions). *Saccharomyces cerevisiae* WE372 was used as the commercial culture for AF inoculation. Fermentations were monitored from the start of fermentation on a weekly basis until completion. Data shown indicate the average changes in the malic acid concentration of each treatment repeated in triplicate. The RSD is less than 10% between fermentation repeats.

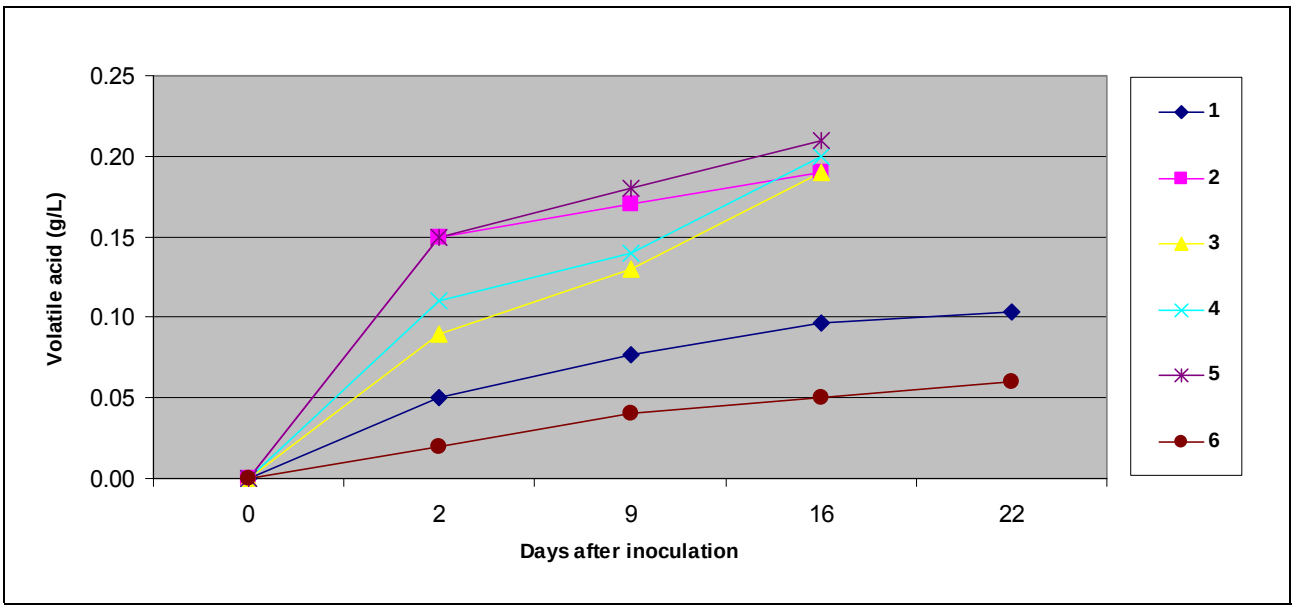

Figure 4.11 The increase in volatile acidity (reported as g/L acetic acid) of the different MLF treatments (refer to **Table 4.4** for treatment descriptions) in Cabernet Sauvignon 2009 under co-inoculation conditions. Each value represents the average of triplicate treatments. The RSD is less than 10% between fermentation repeats.

The microbial populations of the co-inoculated treatments are shown in **Table 4.17**. The mixed LAB combinations (treatments 2, 3 and 4) and VP41 (treatment 5) were inoculated at $4x10^6$ cfu/mL, whilst the spontaneous treatment (1) had an initial bacterial population of $3x10^5$ cfu/mL. The bacterial population in treatments 2, 3 and 4 and the ML01 yeast population (treatment 6) stayed constant until the completion of MLF, whereas the VP41 population decreased slightly towards the end of the fermentation. There was a drastic decrease in the indigenous LAB population in treatment 1, probably due to yeast derived metabolites including ethanol. The cell counts never fully recovered and no spontaneous MLF occurred.

Table 4.17 Cell counts (cfu/mL) of the total LAB population (treatments 1 to 5) and yeast population (treatment 6) of the mixed LAB fermentations as recorded at the start and different stages of MLF in Cabernet Sauvignon 2009 in co-inoculation with *S. cerevisiae* WE372 (treatments 1 to 5). Each enumeration represents the average of triplicate treatments (standard deviations not shown).

	Days after inoculation				
Treatment *	**0**	**2**	**9**	**16**	**22**
1	$3.0x10^5$	nd	nd	$1.3x10^2$	$2.2x10^3$
2	$4.7x10^6$	$2.7x10^6$	$6.0x10^6$	$1.9x10^6$	ndt
3	$4.4x10^6$	$2.4x10^6$	$6.5x10^6$	$2.4x10^6$	ndt
4	$4.2x10^6$	$2.7x10^6$	$3.2x10^6$	$3.4x10^6$	ndt
5	$3.0x10^6$	$9.4x10^6$	$3.4x10^6$	$1.9x10^5$	ndt
6	$1.8x10^7$	$4.8x10^7$	$1.0x10^7$	$2.5x10^6$	$2.7x10^6$

* Refer to **Table 4.4** for treatment descriptions
ndt: not determined as MLF was completed

C) Chardonnay

Inoculation of the treatments after completion of AF was unsuccessful. The total SO_2 concentration was determined and was found to be 50 ppm. This concentration is above levels normally found after the completion of AF and very close to inhibitory concentrations for LAB. The ML01 control treatment contained similar high concentrations of SO_2, which would indicate that the elevated sulphur levels could not be attributed to the presence of the commercial yeast *S. cerevisiae* VIN2000 used for AF in the other treatments, but rather due to inaccurate sulphur additions during processing of the grapes and must. Treatments were re-inoculated for MLF after removal of the yeast lees, but microbiological analysis of the wines showed that there were no bacteria present after inoculation. Treatment of the wine with Malostart (Laffort), a product that mainly consists of yeast hulls to remove potentially harmful metabolites like medium chain fatty acids produced by the yeast, followed by removal of the sediment and re-inoculation, was also unsuccessful. Therefore only co-inoculation results were obtained for Chardonnay in the 2009 vintage.

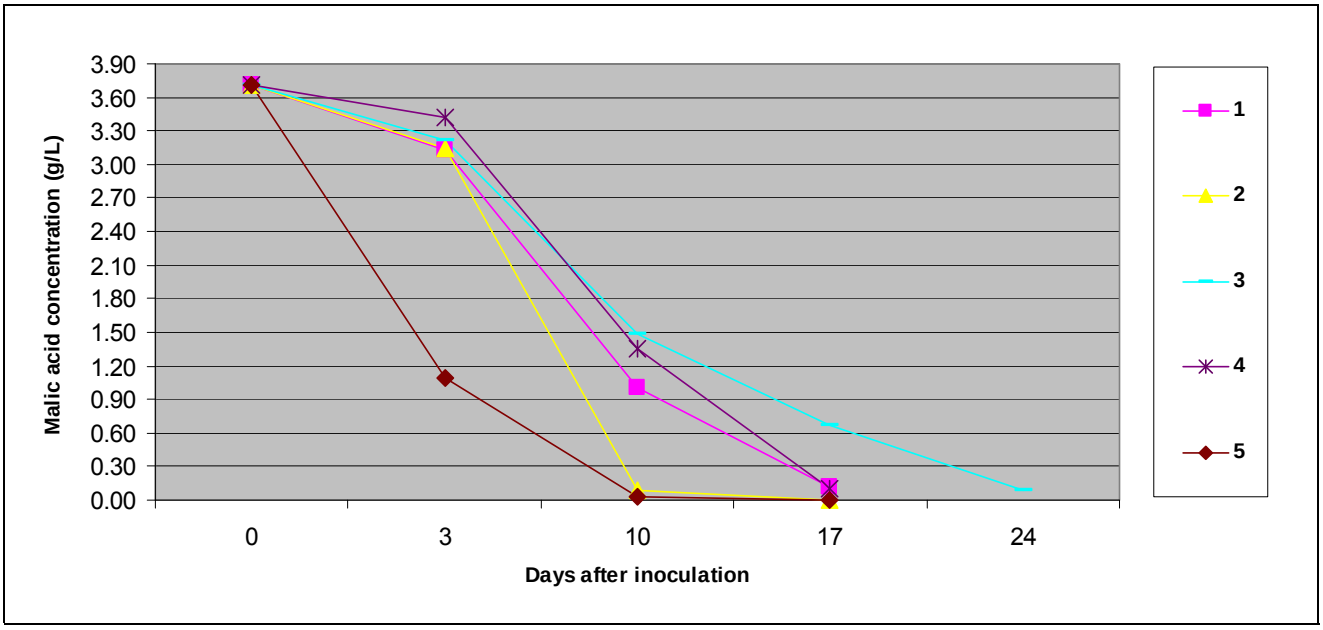

Figure 4.12 A fermentation graph showing the evolution of malic acid (g/L) in the MLF treatments (refer to **Table 4.4** for treatment descriptions) evaluating the mixed strain combinations in Chardonnay 2009 in a co-inoculation scenario. *Saccharomyces cerevisiae* VIN2000 was used as the commercial culture for AF inoculation. Fermentations were monitored from the start of fermentation on a weekly basis until completion. Data shown indicate the average changes in the malic acid concentration of each treatment repeated in triplicate. The RSD is less than 10% between fermentation repeats.

Two of the mixed LAB treatments demonstrated fermentation rates comparable to that of the control fermentation. The first combination (treatment 1), *O. oeni* S5 with *Lb. plantarum* 56, along with the commercial culture VP41, took 17 days to complete MLF. The shortest fermentation period, taking 10 days to complete, was fermentation with *O. oeni* S6 and *Lb. plantarum* 107 (treatment 2) and ML01 (treatment 5), the malolactic wine yeast. The third combination of mixed isolates (treatment 3) took the longest time, 24 days, to complete MLF.

Similar microbial population trends to those found in Pinotage and Cabernet Sauvignon, were observed in the Chardonnay (**Table 4.18**). All the treatments were inoculated at initial cell counts of 10^6 or 10^7 cfu/mL and these stayed constant or slightly increased during the course of the fermentation.

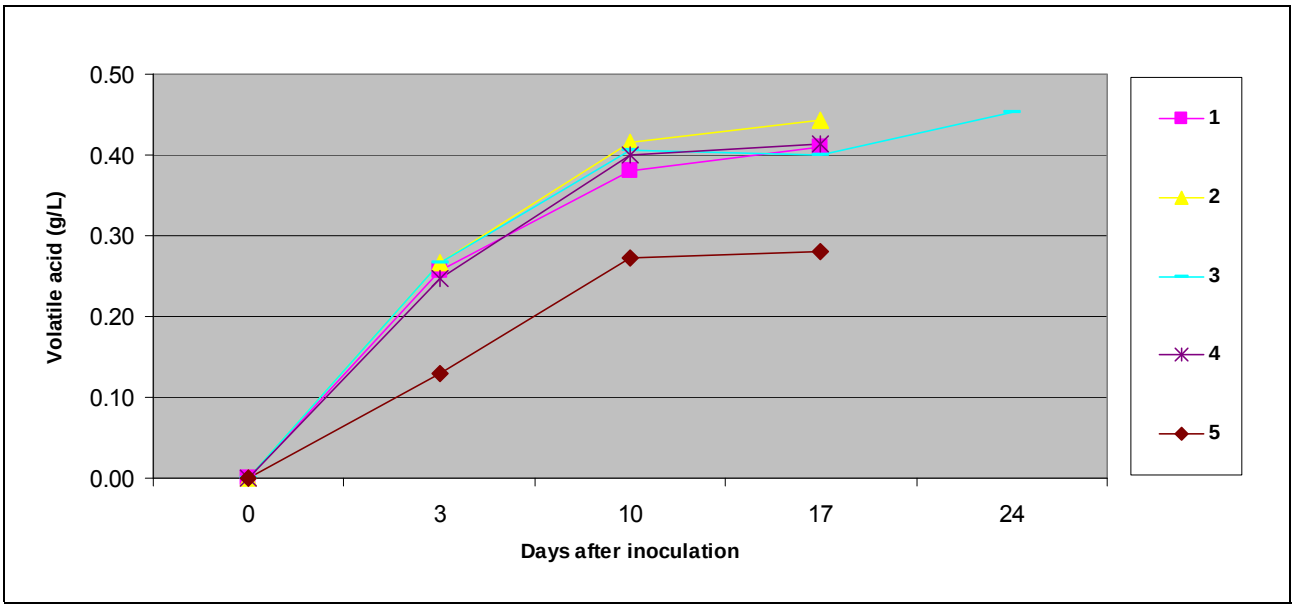

Figure 4.13 The increase in volatile acidity (reported as g/L acetic acid) of the different MLF treatments (refer to **Table 4.4** for treatment descriptions) in Chardonnay 2009 under co-inoculation conditions. Each value represents the average of triplicate treatments. The RSD is less than 10% between fermentation repeats.

Table 4.18 Cell counts (cfu/mL) of the total LAB population (treatments 1 to 4) and yeast population (treatment 5) of the mixed LAB fermentations as recorded at the start and different stages of MLF in Chardonnay 2009 in co-inoculation with *S. cerevisiae* VIN2000 (treatments 1 to 4). Each enumeration represents the average of triplicate treatments (standard deviations not shown).

	Days after inoculation				
Treatment *	**0**	**3**	**10**	**17**	**24**
1	$3.8x10^6$	$4.7x10^6$	$5.0x10^6$	$8.0x10^6$	ndt
2	$5.3x10^6$	$8.0x10^6$	$2.6x10^7$	$2.5x10^6$	ndt
3	$3.4x10^6$	$6.0x10^6$	$7.6x10^6$	$8.9x10^6$	$6.1x10^6$
4	$1.1x10^6$	$1.5x10^6$	$1.5x10^7$	$2.1x10^6$	ndt
5	$1.3x10^7$	$3.9x10^6$	$5.0x10^6$	$4.8x10^6$	$2.6x10^6$

* Refer to **Table 4.4** for treatment descriptions
ndt: not determined as MLF was completed

The mixed LAB cultures are clearly compatible with the two commercial yeast strains used in this study, *S. cerevisiae* WE372 and *S. cerevisiae* VIN2000. It is necessary to investigate the compatibility of these bacterial combinations with other commonly used commercial yeast cultures. The two inoculation scenarios, co-inoculation and sequential inoculation, both resulted in the successful completion of MLF with no excessive amounts of volatile acid being produced. MLF with

the mixed cultures were successful in Pinotage, Cabernet Sauvignon and Chardonnay, but further research is needed, focusing on other red wine cultivars that are also subjected to MLF.

4.3.2 PRODUCTION OF VOLATILE AROMA COMPOUNDS

MLF, via the metabolic activities of LAB, has the ability to alter the aroma profile of a wine by the production or modification of flavour-active compounds (Lonvaud-Funel, 1999; Swiegers *et al*., 2005). There are three mechanisms by which LAB are able to modify the wine aroma profile. LAB are able to produce volatile compounds by the metabolism of grape-derived components, by the modification of grape- or yeast-derived secondary metabolites and by the adsorption of compounds to the cell wall or the metabolism of flavour compounds (Bartowsky and Henschke, 1995). The general flavour changes associated with MLF include wines having better mouthfeel properties, having a creamier palate and more butteriness and reduced vegetative, green and grassy aromas. Fruity notes seem to be either enhanced or diminished by MLF (Henick-Kling, 1993; Bartowsky *et al*., 2002; Liu, 2002; Jeromel *et al*., 2008). The changes in aroma and flavour profiles during MLF are also dependant on the bacteria strain (Bartowsky and Henschke, 1995; Costello, 2006), the grape cultivar and winemaking practises (Bartowsky *et al*., 2002). At present it has been established that LAB are capable of producing, modifying or metabolising various volatile aroma compounds such as esters, higher alcohols, volatile fatty acids and nitrogen- and sulphur-containing compounds, as well as one of the most sensorial significant compounds produced during MLF, diacetyl, which is responsible for the buttery notes in wine (Swiegers *et al*., 2005).

In the present study, the concentrations of most volatile compounds were higher in one cultivar than in the others, or even absent in one of the cultivars and produced in others. In general, MLF treatments in Cabernet Sauvignon 2008 produced lower concentrations of total esters and volatile acids, whilst lower concentrations of total alcohols were produced in Shiraz 2008. The only apparent difference in volatile compounds due to inoculation time is the production of higher total acid concentrations in the co-inoculated Pinotage 2009 compared to the sequential inoculation. Similar to results obtained in 2008, MLF in Cabernet Sauvignon 2009 produced lower levels of total acids and also higher total alcohol concentrations. However, due to the fact that the production of these volatile compounds are dependant on the LAB treatment, cultivar and vintage, the compounds that exhibit noticeable trends between different MLF treatments will be the focus and discussed individually with reference to cultivar (**Tables 4.19** to **4.25**). GC-FID and GC-MS generated data were not subjected to univariate statistical analysis and results discussed only refer to trends observed in the data.

4. 3.2.1 2008

The wines from the 2008 vintage were subjected to GC-FID analysis to ascertain the changes in the volatile composition that could be attributed to the different bacterial combinations. These

results were used to investigate the trends in the volatile profiles of the wines. Results obtained were, in conjunction with results from the informal tasting, also utilised in selecting the combinations of LAB cultures to be evaluated in the 2009 vintage. Aroma descriptors associated with the volatile compounds are indicated in parenthesis in the text.

4.3.2.1.1 Esters

Esters are important compounds in defining fruity aromas and can be grape derived or form via the esterification of acids and alcohols due to esterase associated activity (Matthews *et al*., 2004). Esters are fermentative products and include ethyl esters of organic acids, ethyl esters of fatty acids and acetate esters from higher alcohols. The extent to which LAB are able to alter the ester concentration during MLF is still unclear. Both increase and decrease in ester concentrations during MLF have been reported in the literature (Maicas *et al*., 1999; Matthews *et al*., 2004; Ugliano and Moio, 2005). The final ester concentration is also dependant on the strain of LAB that conduct the fermentation and esterase from *O. oeni* and other LAB genera, have been screened and characterised (Davis *et al*., 1988; Matthews *et al*., 2006; Mtshali *et al*., 2009).

MLF in Pinotage (**Table 4.19**) was generally associated with an increase in the ethyl esters, excluding ethyl butyrate and ethyl hexanoate, and a decrease in the acetate ester concentrations. Ethyl acetate was quantitatively the predominant ester after the completion of MLF. At lower concentrations it adds to wine complexity and imparts pleasant and fruity aromas, while higher concentrations are detrimental to wine quality and associated with descriptors like nail polish remover. Ethyl acetate decreased during MLF in the inoculated treatments, while the smallest decrease was observed in treatments 7, 8 and 9 (treatments containing *Lb. plantarum* 107 as part of the mixed culture) and the control treatment 10. Isoamyl acetate (banana), 2-phenylethyl acetate (rose, fruity) and hexyl acetate (sweet, fruity) decreased in the inoculated treatments and hexyl acetate was not detected in any of the three cultivars in the 2008 vintage. Ethyl butyrate (fruity, pineapple) and ethyl hexanoate (green apple, fruity, violets) slightly decreased over the course of the fermentation. Ethyl caprylate (apple) increased in the inoculated treatments with the exception of treatments 8, 9 and 10, whilst ethyl caprate (fruity) was always higher in the treatments that had undergone MLF. Two of the most important esters that typically play a role in MLF, are ethyl lactate and diethyl succinate (Maicas *et al*., 1999; Herjavec *et al*., 2001; Ugliano and Moio, 2005). Ethyl lactate is an important flavour compound formed during MLF via the esterification of ethanol and lactic acid and increased concentrations are produced during MLF as a result of increased lactate concentrations. This compound contributes to the buttery, milky, sweet and strawberry aromas and smooth mouthfeel characteristics of the wine. Increased concentrations were evident in six of the nine treatments, with the exception of treatments 3, 6 and 9. This lack of ethyl lactate production could be attributed to the specific *O. oeni* strain present in all three treatments. This trend was observed in all three cultivars (**Figure 4.14**). Diethyl succinate (fruity, melon) also increased during MLF, with the smallest increase evident in treatments 3 and 9.

Table 4.19 Esters, higher alcohols and volatile fatty acids (mg/L) measured in 2008 in Pinotage after MLF with mixed isolates. Concentrations represent the average of triplicate treatment repeats, each analysed in triplicate by GC-FID (standard deviations not shown) (nd: not detected).

Treatments*	before MLF	1	2	3	4	5	6	7	8	9	10	11
Esters												
Ethyl Butyrate	0.707	0.619	0.628	0.621	0.602	0.629	0.637	0.620	0.629	0.626	0.644	0.524
Ethyl Hexanoate	0.679	0.619	0.624	0.621	0.624	0.621	0.648	0.605	0.623	0.575	0.538	0.497
Ethyl Lactate	nd	20.015	21.117	nd	19.053	24.373	nd	32.481	23.346	nd	12.144	0.457
Ethyl Caprylate	0.235	0.264	0.265	0.270	0.277	0.274	0.292	0.267	nd	nd	nd	0.185
Ethyl Caprate	nd	0.083	0.083	0.088	0.090	0.087	0.102	0.086	0.093	0.084	0.082	nd
Diethyl Succinate	0.370	0.612	0.596	0.578	0.626	0.711	0.626	0.631	0.635	0.551	0.603	0.529
Ethyl Acetate	131.332	87.258	92.085	86.366	79.918	87.833	86.020	122.272	97.358	114.457	111.880	129.517
Isoamyl Acetate	4.014	2.963	3.092	2.904	2.870	2.903	3.134	2.945	3.077	2.828	2.456	2.280
Hexyl Acetate	0.377	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-Phenylethyl Acetate	0.049	0.032	0.031	0.033	0.034	0.041	0.041	0.021	0.040	0.029	0.036	0.006
Total	**137.763**	**112.465**	**118.521**	**91.481**	**104.094**	**117.472**	**91.5**	**159.928**	**125.801**	**119.15**	**128.383**	**133.995**
Alcohols												
Methanol	35.023	140.386	146.243	138.508	136.518	157.450	136.520	149.032	156.014	183.642	162.409	332.426
Propanol	116.625	114.557	117.406	112.745	108.106	122.584	112.763	171.298	125.175	157.261	133.929	239.362
Isobutanol	24.121	22.267	22.613	22.173	21.351	23.810	22.554	31.060	24.481	29.362	29.099	36.0268
Butanol	2.009	1.886	1.873	1.870	1.822	2.009	1.881	2.525	2.042	2.405	2.418	2.836
Isoamyl alcohol	180.397	169.947	168.493	168.666	165.214	184.176	176.761	193.098	183.612	193.976	187.802	195.378
Hexanol	0.833	0.853	0.835	0.829	0.859	0.872	0.873	0.857	0.868	0.785	0.868	0.625
2-Phenyl Ethanol	22.066	20.751	20.197	20.625	20.413	21.650	21.453	23.356	22.840	23.223	23.201	23.167
Total	**381.074**	**470.647**	**477.66**	**465.416**	**454.283**	**512.551**	**472.805**	**648.227**	**515.032**	**590.654**	**539.726**	**829.821**
Volatile Fatty Acids												
Acetic acid	288.205	397.483	384.981	343.828	361.901	418.457	380.503	572.551	406.973	446.028	366.128	632.716
Propionic Acid	9.300	9.097	8.896	8.331	8.201	8.838	7.779	12.465	8.817	9.826	9.896	15.965
Iso-Butyric Acid	0.656	0.660	0.639	0.664	0.633	0.691	0.701	0.808	0.772	0.825	0.785	0.888
Butyric Acid	1.320	1.304	1.283	1.291	1.283	1.386	1.360	1.582	1.539	1.586	1.506	1.747
Iso-Valeric Acid	0.601	0.636	0.623	0.631	0.624	0.693	0.673	0.729	0.714	0.714	0.755	0.839
Valeric Acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Hexanoic Acid	1.560	1.607	1.606	1.616	1.652	1.685	1.763	1.575	1.618	1.501	1.647	1.212
Octanoic Acid	1.269	1.523	1.523	1.534	1.571	1.674	1.663	1.356	1.650	1.347	1.534	0.949
Decanoic Acid	0.248	0.394	0.392	0.409	0.410	0.423	0.438	0.388	0.394	0.377	0.407	0.258
Total	**303.159**	**412.704**	**399.943**	**358.304**	**376.275**	**433.847**	**394.88**	**591.454**	**422.477**	**462.204**	**382.658**	**654.574**

* Refer to **Table 4.3** for a description of the treatments

The overall trend that seemed to emerge was that lower concentrations of total esters, specifically ethyl lactate and diethyl succinate, were produced by the treatments containing *O. oeni* E53 as part of the mixed culture (treatments 3, 6 and 9).

Contrary to Pinotage, ethyl lactate was the quantitatively predominant ester after MLF in Cabernet Sauvignon (**Table 4.20**). This is due to the higher initial malic acid concentration in Cabernet Sauvignon and the concomitant lactic acid produced during MLF. Similar concentrations were produced by six of the treatments, with lower concentrations produced by treatments 3, 6 and 9 as previously mentioned. The six treatments with the mixed isolates produced higher concentrations of ethyl lactate compared to treatment 10, the commercial culture. Esters that were not greatly affected by MLF, with the exception of treatments 3, 6 and 9 that led to an increased production of these esters (**Figure 4.15**), were ethyl hexanoate, ethyl caprylate, ethyl caprate, isoamyl acetate and diethyl succinate. Concentrations of some of the fruity esters produced by treatments 3, 6 and 9 were at levels well above the threshold values. Similar concentrations of ethyl butyrate and ethyl acetate were produced by the inoculated treatments while no detectable levels of hexyl acetate or 2-phenylethyl acetate were produced. Similar to the trend observed in Pinotage, treatments 3, 6 and 9 produced lower levels of total esters, but still produced increased concentrations of the fruity esters.

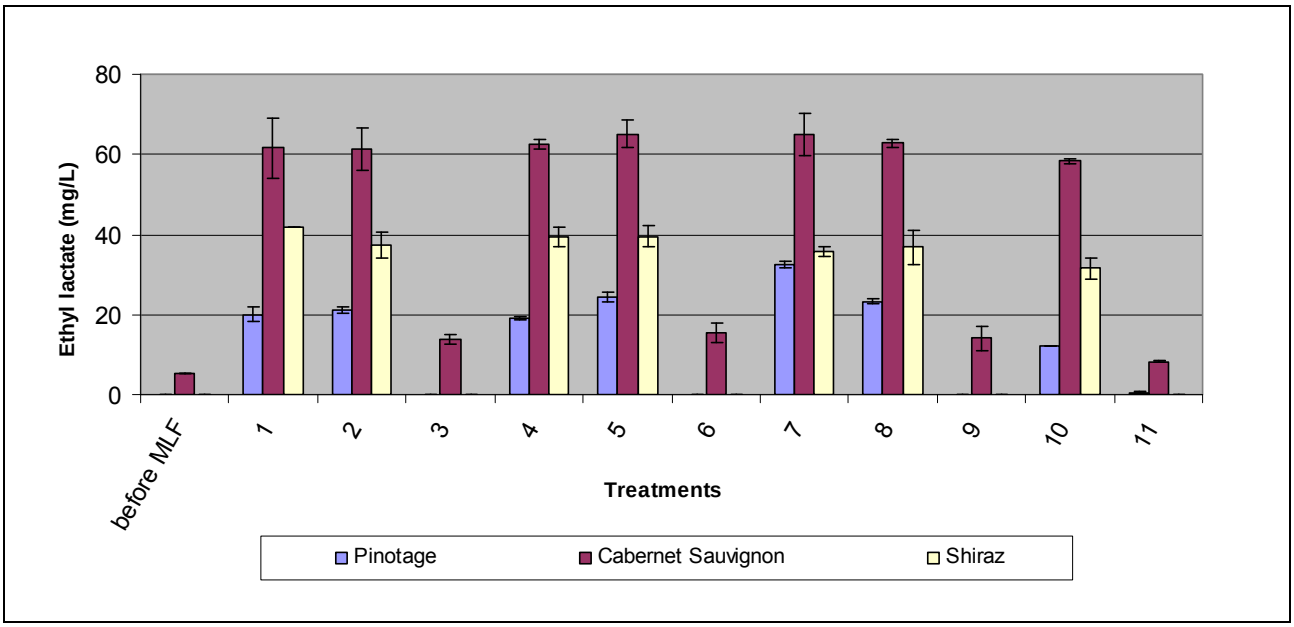

Figure 4.14 The ethyl lactate (mg/L) production by the MLF treatments (refer to **Table 4.3** for treatment descriptions) observed in Pinotage, Cabernet Sauvignon and Shiraz in the 2008 vintage in sequential inoculation. Each value represents the average of triplicate treatments.

Similar to results obtained in both Pinotage and Cabernet Sauvignon, treatments 3, 6 and 9 produced lower concentrations of total esters and no detectable quantities of ethyl lactate in the Shiraz wines (**Table 4.21**). No discernable trends were evident for the remaining esters, with similar quantities being produced by the remaining treatments for the majority of the esters.

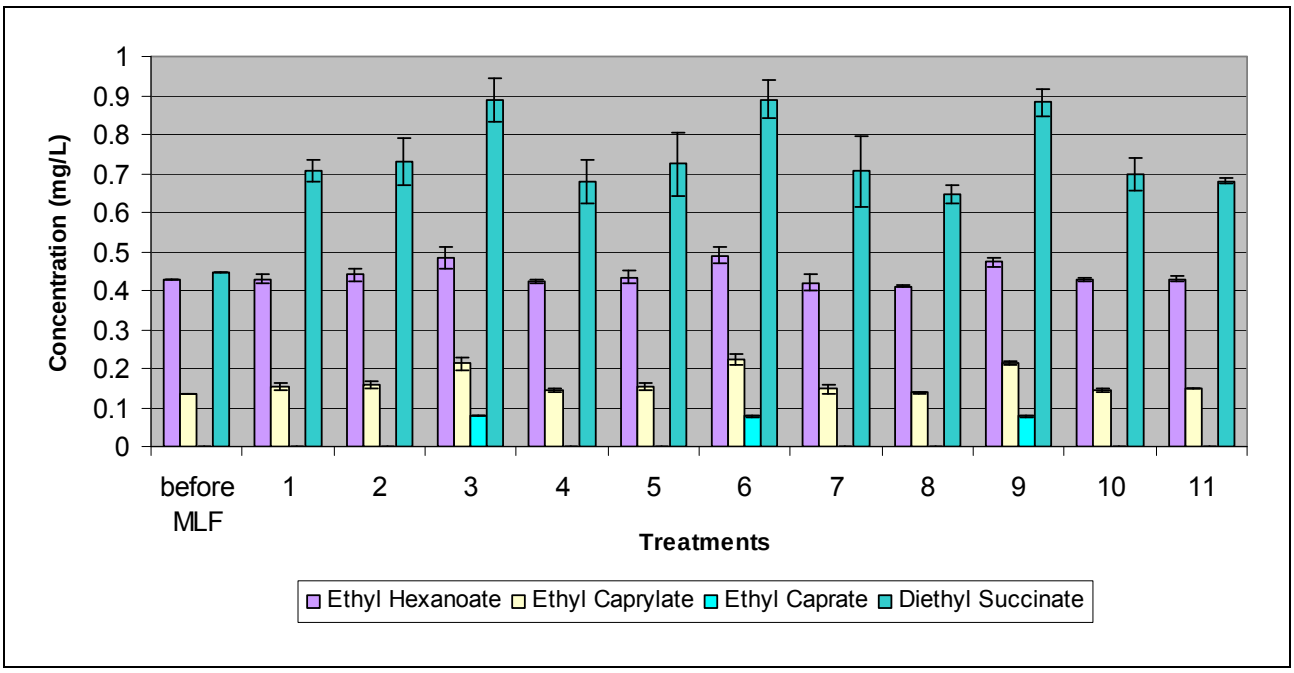

Figure 4.15 The ester (mg/L) production by the MLF treatments (refer to **Table 4.3** for treatment descriptions) observed in Cabernet Sauvignon in the 2008 vintage in sequential inoculation. Each value represents the average of triplicate treatments. Higher concentrations of the fruity esters were produced by treatments 3, 6 and 9.

4.3.2.1.2 Alcohols

Higher alcohols, also known as fusel alcohols, are alcohols that contain more than two carbon molecules. At lower concentrations (less than 300 mg/L), higher alcohols can potentially add to wine complexity and fruity aromas, whereas higher concentrations (above 400 mg/L) could potentially be unfavourable and impart harsh, chemical-like aromas (Swiegers *et al*., 2005). Maicas *et al*. (1999) found the production of higher alcohols to be strain dependant, while Pozo-Bayón *et al.* (2005) only observed insignificant increases in the levels of higher alcohols after MLF. The fact that literature suggests that LAB have limited ability to produce these fusel alcohols, could be beneficial in avoiding the production of harsh, solvent-like aroma compounds.

In Pinotage, the highest concentration of propanol (pungent, harsh, ripe fruit), isobutanol (fusel, spirituous), butanol (fusel, alcoholic, medicinal), isoamyl alcohol (harsh, nail polish) and 2-phenyl ethanol (floral, rose, honey, spice, lilac) were observed in treatments 7, 8, 9 and 10. These included the treatments with *Lb. plantarum* 107 as part of the mixed culture and the commercial starter culture. These four treatments also contained the highest total alcohol concentration.

Table 4.20 Esters, higher alcohols and volatile fatty acids (mg/L) measured in 2008 in Cabernet Sauvignon after MLF with mixed isolates. Concentrations represent the average of triplicate treatment repeats, each analysed in triplicate by GC-FID (standard deviations not shown) (nd: not detected).

Treatments *	before MLF	1	2	3	4	5	6	7	8	9	10	11
Esters												
Ethyl Butyrate	0.393	0.403	0.403	0.414	0.382	0.393	0.403	0.384	0.384	0.405	0.397	0.402
Ethyl Hexanoate	0.429	0.428	0.441	0.483	0.423	0.435	0.491	0.421	0.411	0.473	0.426	0.430
Ethyl Lactate	5.291	66.019	58.190	13.803	62.554	65.175	15.465	61.911	62.781	14.141	58.359	8.179
Ethyl Caprylate	0.133	0.153	0.158	0.213	0.144	0.155	0.224	0.147	0.138	0.212	0.144	0.150
Ethyl Caprate	nd	nd	nd	0.079	nd	nd	0.078	nd	nd	0.078	nd	nd
Diethyl Succinate	0.447	0.708	0.729	0.888	0.680	0.724	0.890	0.706	0.646	0.882	0.698	0.681
Ethyl Acetate	37.210	33.514	34.577	37.523	30.745	32.115	32.937	29.730	32.979	29.637	35.666	35.769
Isoamyl Acetate	0.983	0.967	0.996	1.075	0.896	0.965	1.068	0.894	0.874	1.010	0.964	0.961
Hexyl Acetate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-Phenylethyl Acetate	nd	nd	nd	0.001	nd	nd	0.003	nd	nd	nd	nd	nd
Total	**44.886**	**102.192**	**95.494**	**54.479**	**95.824**	**99.962**	**51.559**	**94.193**	**98.213**	**46.838**	**96.654**	**46.572**
Alcohols												
Methanol	181.233	156.830	150.260	195.731	152.890	152.847	178.787	155.012	163.959	188.664	176.171	163.811
Propanol	47.172	46.946	45.582	53.432	43.178	44.562	53.355	42.396	46.641	49.439	49.742	47.035
Isobutanol	33.166	31.615	31.099	35.767	29.553	31.214	36.516	29.564	32.270	36.749	33.392	32.519
Butanol	1.997	2.077	1.915	2.284	1.950	2.047	2.179	1.900	1.950	2.140	2.143	2.062
Isoamyl alcohol	371.582	371.893	357.114	425.365	362.722	393.370	406.633	355.950	367.010	414.890	387.791	378.563
Hexanol	1.013	1.108	1.158	1.247	1.087	1.175	1.332	1.128	1.062	1.205	1.153	1.082
2-Phenyl Ethanol	90.723	90.930	89.318	102.029	89.448	95.956	102.219	89.292	89.582	100.444	94.578	92.868
Total	**726.886**	**701.399**	**676.446**	**815.855**	**680.828**	**721.171**	**781.021**	**675.242**	**702.474**	**793.531**	**744.970**	**717.940**
Volatile Fatty Acids												
Acetic acid	106.926	214.333	201.081	228.866	210.727	213.494	253.097	209.529	208.509	245.039	178.142	108.886
Propionic Acid	2.322	3.458	3.112	3.577	3.123	3.350	3.608	3.255	3.013	3.420	3.399	3.117
Iso-Butyric Acid	1.212	1.229	1.250	1.453	1.227	1.322	1.540	1.299	1.251	1.387	1.318	1.343
Butyric Acid	0.676	0.678	0.686	0.755	0.681	0.728	0.809	0.710	0.679	0.710	0.723	0.696
Iso-Valeric Acid	2.399	2.426	2.500	2.818	2.395	2.585	2.999	2.544	2.426	2.699	2.544	2.493
Valeric Acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Hexanoic Acid	0.917	0.969	0.984	1.073	0.920	0.997	1.133	0.964	0.899	1.028	0.977	0.937
Octanoic Acid	0.825	0.897	0.908	0.965	0.849	0.901	1.010	0.883	0.842	0.945	0.892	0.815
Decanoic Acid	0.248	0.304	0.320	0.593	0.319	0.327	0.608	0.322	0.308	0.547	0.334	0.260
Total	**115.525**	**224.294**	**210.841**	**240.100**	**220.241**	**223.704**	**264.804**	**219.506**	**217.933**	**255.775**	**188.336**	**118.547**

* Refer to **Table 4.3** for a description of the treatments

Table 4.21 Esters, higher alcohols and volatile fatty acids (mg/L) measured in 2008 in Shiraz after MLF with the mixture of isolates. Concentrations represent the average of triplicate treatment repeats, each analysed in triplicate by GC-FID (standard deviations not shown) (nd: not detected).

Treatments *	before MLF	1	2	3	4	5	6	7	8	9	10	11
Esters												
Ethyl Butyrate	0.497	0.463	0.502	0.517	0.540	0.493	0.536	0.539	0.532	0.529	0.527	0.521
Ethyl Hexanoate	0.485	0.447	0.511	0.525	0.538	0.518	0.527	0.547	0.531	0.530	0.540	0.536
Ethyl Lactate	nd	41.832	37.196	nd	39.356	39.572	nd	35.775	36.777	nd	31.499	nd
Ethyl Caprylate	0.175	0.207	0.209	0.216	0.227	0.211	0.208	0.228	0.217	0.209	0.220	0.206
Ethyl Caprate	nd	0.078	0.075	0.075	0.077	0.075	0.074	0.076	nd	nd	nd	nd
Diethyl Succinate	0.412	0.214	0.227	0.226	0.273	0.250	0.265	0.293	0.300	0.283	0.274	0.270
Ethyl Acetate	103.687	72.083	64.620	62.617	61.418	59.500	67.426	60.746	64.938	65.525	61.328	59.612
Isoamyl Acetate	1.858	2.392	2.505	2.596	2.686	2.504	2.618	2.724	2.624	2.624	2.683	2.685
Hexyl Acetate	nd	nd	0.123	0.123	0.371	0.246	0.251	0.370	0.247	0.123	0.061	0.243
2-Phenylethyl Acetate	0.019	0.080	0.078	0.085	0.088	0.080	0.095	0.093	0.083	0.088	0.093	0.094
Total	**107.133**	**169.543**	**106.046**	**66.980**	**105.574**	**103.449**	**72.000**	**101.391**	**106.249**	**69.870**	**97.225**	**64.167**
Alcohols												
Methanol	221.138	250.797	201.767	189.010	230.657	241.501	251.187	207.481	218.477	223.510	205.540	194.217
Propanol	171.890	105.664	98.319	94.340	94.615	92.266	98.404	95.912	96.047	105.365	97.287	97.054
Isobutanol	39.934	28.226	25.872	25.184	25.783	25.191	27.124	25.405	26.847	27.588	25.329	25.335
Butanol	3.194	2.428	2.216	2.199	2.296	2.261	2.312	2.217	2.324	2.400	2.183	2.207
Isoamyl alcohol	238.151	217.182	211.164	212.092	215.387	217.445	205.467	207.435	207.279	219.647	203.833	205.291
Hexanol	0.834	0.895	0.972	0.956	1.049	1.012	0.978	1.068	1.070	1.026	1.079	0.991
2-Phenyl Ethanol	31.018	30.808	27.383	27.616	28.645	28.996	28.148	28.096	28.901	28.808	27.259	27.699
Total	**706.159**	**636.000**	**567.693**	**551.397**	**598.432**	**608.672**	**613.620**	**567.614**	**580.945**	**608.344**	**562.510**	**552.794**
Volatile Fatty Acids												
Acetic acid	285.291	457.855	450.337	337.557	460.317	456.267	312.842	421.842	404.455	356.886	416.183	302.569
Propionic Acid	9.455	6.868	6.061	5.688	6.905	7.252	6.240	6.184	6.558	6.573	5.840	6.210
Iso-Butyric Acid	1.140	0.951	0.888	0.924	0.914	0.957	0.958	0.891	0.930	0.991	0.879	0.947
Butyric Acid	1.262	1.159	0.945	0.946	0.982	1.014	0.966	0.944	0.990	1.029	0.956	0.997
Iso-Valeric Acid	0.880	0.977	0.908	0.926	0.963	0.950	0.953	0.950	0.962	0.969	0.928	0.949
Valeric Acid	nd	0.069	0.179	0.267	0.289	0.305	0.281	0.273	0.279	0.287	0.258	0.262
Hexanoic Acid	1.093	1.092	1.250	1.297	1.355	1.301	1.340	1.395	1.369	1.337	1.396	1.360
Octanoic Acid	0.919	1.069	1.276	1.306	1.404	1.306	1.314	1.448	1.393	1.317	1.431	1.274
Decanoic Acid	0.280	0.322	0.358	0.382	0.384	0.365	0.380	0.385	0.374	0.370	0.380	0.337
Total	**300.32**	**470.362**	**462.202**	**349.293**	**473.513**	**469.717**	**325.274**	**434.312**	**417.31**	**369.759**	**428.251**	**314.905**

* Refer to **Table 4.3** for a description of the treatments

Contrary to the results observed in Pinotage, treatments 3, 6 and 9 in Cabernet Sauvignon, produced the highest total alcohol concentrations, as well as the highest concentrations of all the individual alcohols, with isoamyl alcohol being the most abundant alcohol present after MLF.

Very few trends and differences were observed in the alcohol concentrations in Shiraz after MLF. Treatments 4, 5 and 6 (treatments containing *Lb. plantarum* 56) seem to have slightly higher total alcohol concentrations, but differences between treatments are small and most likely insignificant, as confirmed by results from Ugliano and Moio (2005).

4.3.2.1.3 Volatile Fatty Acids

Volatile fatty acids are produced as products of the fatty acid metabolism of yeast and bacteria. Results observed in literature regarding the concentrations of volatile fatty acids associated with LAB metabolism hold opposing views. Both increases and decreases have been reported and could imply that the predilection for producing these acids, is more than likely strain dependant (Maicas *et al*., 1999; Herjavec *et al*., 2001).

In Pinotage, concentrations of acetic acid (vinegar), propionic acid (pungent, vinegar), iso-butyric acid (rancid, cheese), butyric acid (cheese, sweaty) and iso-valeric acid (cheese, sweaty) were the highest in treatments 7, 8 and 9. These treatments contained *Lb. plantarum* 107 as part of the mixed culture. Valeric acid (cheese, sweaty) was not detectable in any of the treatments and this result was also observed in Cabernet Sauvignon, with the only detectable concentrations being observed in Shiraz. No discernable trends were visible for hexanoic acid (rancid, cheese, sweaty, metallic). Concentrations of the medium chain fatty acids octanoic (oily, sweaty, rancid, sweet, faint fruity) and decanoic acid (rancid, fatty, phenolic, citrus), seem to be the highest in treatments 4, 5 and 6. These treatments contained *Lb. plantarum* 56 as part of the mixed culture.

The most obvious trend observed in Cabernet Sauvignon was the higher concentrations of most of the volatile acids, with the exception of valeric acid, in treatments 3, 6 and 9. The highest total fatty acid concentrations associated with these three treatments could be ascribed to the presence of *O. oeni* E53. Contrary to the results observed in Cabernet Sauvignon, the lowest total concentration of fatty acids in Shiraz was observed in treatments 3, 6 and 9.

It is clear from the results obtained in this study that different bacterial strains and mixed cultures can result in different aroma profiles in the final wines. It appears that the final ester concentration could be attributed to the *O. oeni* strain, rather than the *Lb. plantarum* strain, present in the mixed culture. In two of the three cultures, the presence of a specific *Lb. plantarum* strain was responsible for the differences observed in the higher alcohol concentrations. The opposite was observed for the volatile acids, where in two of the cultivars, the final total acid concentrations could be linked to the specific *O. oeni* strain present in the mixed culture. It also appears as if the presence of the specific *Lb. plantarum* strain had a greater role to play in the Pinotage aroma profile, compared to the effect of the *O. oeni* strain that seems to be more pronounced in Cabernet

Sauvignon and Shiraz. The sensory thresholds of the volatile compounds should also be taken into account, in order to clearly elucidate the resulting affect that these compounds will have on the wine aroma profile. In order to clearly understand the effect that LAB have on the final wine aroma, other factors including cultivar variance and inoculation times should also be considered.

4.3.2.2 2009

The wines from the 2009 vintage were subjected to GC-FID analysis to ascertain the changes in the volatile composition that could be attributed to the different bacterial combinations as well as GC-MS analysis for carbonyl compounds. These results were used to investigate trends in the volatile profile of the wines.

4.3.2.2.1 Esters

There were noted differences between most of the ester concentrations produced by the different LAB in the Pinotage co-inoculation treatments (**Table 4.22**), with the exception of ethyl acetate, 2-phenylethyl acetate and hexyl acetate. The lowest level of total esters were observed for treatment 6, the ML01 yeast, while similar levels of total esters were produced by treatments 1, 2, 3 and 5. Similar to results obtained in the previous vintage, treatment 4, containing *O. oeni* E53 as part of the mixed culture, produced the lowest concentration of total esters, with the most noted difference in the lower levels of ethyl lactate. The other two mixed cultures, treatments 2 and 3, contained higher concentrations of total esters after the completion of MLF. Similar concentrations of esters were produced by the indigenous LAB in treatment 1 and the commercial culture, treatment 5.

In results similar to that observed for co-inoculation, the lowest total ester concentration found in the sequentially inoculated treatments, were associated with treatment 4 (**Table 4.23**). The other two mixed cultures, treatments 2 and 3, as well as treatment 1, showed similar changes in the ester profile. The MLF treatments did not seem to differ in their ability to alter the concentrations of ethyl butyrate, ethyl hexanoate, ethyl caprate, ethyl caprylate, ethyl acetate, isoamyl acetate and hexyl acetate. The biggest differences in the total ester concentrations seem to be attributed to the difference in ethyl lactate and diethyl succinate production. Similar concentrations of ethyl lactate were produced by treatments 1, 2 and 3, while the highest and lowest concentrations were associated with treatments 5 and 4, respectively. Likewise, treatments 2 and 3 produced similar diethyl succinate concentrations, while the highest and lowest concentrations were associated with treatments 1 and 4, respectively.

In general, sequential inoculation in Pinotage seem to lead to an overall decrease in the ester concentrations produced during MLF, most notably due to lower levels of ethyl lactate, diethyl succinate, ethyl acetate and 2-phenylethyl acetate produced by some of the

Table 4.22 Esters, higher alcohols and volatile fatty acids (mg/L) measured in 2009 in Pinotage (co-inoculation) after MLF with the selected combinations of mixed LAB. Concentrations represent the average of triplicate treatment repeats, each analysed in triplicate by GC-FID. Standard deviations are shown (95% confidence interval).

Treatments *	1	2	3	4	5	6
Esters						
Ethyl Butyrate	0.858 ± 0.00	0.827 ± 0.01	0.843 ± 0.00	0.874 ± 0.02	0.834 ± 0.01	0.826 ± 0.00
Ethyl Hexanoate	1.443 ± 0.00	1.408 ± 0.01	1.451 ± 0.01	1.465 ± 0.01	1.414 ± 0.01	1.405 ± 0.02
Ethyl Lactate	43.326 ± 1.22	59.721 ± 0.11	55.451 ± 2.49	28.620 ± 2.15	51.057 ± 3.80	30.168 ± 0.73
Ethyl Caprylate	0.288 ± 0.03	0.275 ± 0.03	0.300 ± 0.03	0.279 ± 0.03	0.261 ± 0.01	0.263 ± 0.01
Ethyl Caprate	0.254 ± 0.00	0.250 ± 0.00	0.260 ± 0.01	0.255 ± 0.00	0.258 ± 0.00	0.253 ± 0.00
Diethyl Succinate	1.095 ± 0.10	1.010 ± 0.06	0.886 ± 0.05	0.935 ± 0.01	1.205 ± 0.04	1.424 ± 0.04
Ethyl Acetate	45.164 ± 5.76	42.901 ± 4.78	42.584 ± 4.53	43.077 ± 4.68	38.306 ± 1.21	31.916 ± 1.34
Isoamyl Acetate	1.565 ± 0.04	1.458 ± 0.02	1.452 ± 0.01	1.488 ± 0.02	1.435 ± 0.03	1.409 ± 0.00
Hexyl Acetate	0.939 ± 0.00	1.140 ± 0.05	0.944 ± 0.01	0.933 ± 0.01	1.015 ± 0.09	0.939 ± 0.01
2-Phenylethyl Acetate	1.169 ± 0.01	1.165 ± 0.00	1.165 ± 0.01	1.162 ± 0.00	1.163 ± 0.00	1.158 ± 0.00
Total	**96.101**	**110.155**	**105.336**	**79.088**	**96.948**	**69.761**
Alcohols						
Methanol	38.347 ± 2.65	36.872 ± 2.56	36.901 ± 2.65	38.200 ± 3.28	39.070 ± 1.27	34.123 ± 4.26
Propanol	46.864 ± 0.31	37.939 ± 0.16	40.514 ± 0.70	46.951 ± 2.54	49.507 ± 1.24	31.536 ± 3.18
Isobutanol	21.141 ± 0.77	21.173 ± 0.99	19.343 ± 1.24	21.583 ± 3.13	22.923 ± 0.36	33.360 ± 3.73
Butanol	0.972 ± 0.05	0.940 ± 0.09	0.936 ± 0.04	0.921 ± 0.07	0.981 ± 0.04	0.867 ± 0.02
Isoamyl alcohol	150.505 ± 0.60	159.069 ± 3.78	147.494 ± 2.65	149.978 ± 2.52	154.822 ± 1.34	154.861 ± 3.53
Hexanol	2.156 ± 0.00	2.151 ± 0.01	2.159 ± 0.02	2.199 ± 0.03	2.209 ± 0.00	2.192 ± 0.00
2-Phenyl Ethanol	28.669 ± 1.71	29.569 ± 0.55	27.193 ± 1.15	29.645 ± 1.54	29.547 ± 0.82	31.194 ± 1.95
Total	**288.654**	**287.713**	**274.540**	**289.477**	**299.059**	**288.133**
Volatile Fatty Acids						
Acetic acid	232.620 ± 16.24	261.106 ± 15.15	266.370 ± 7.09	299.361 ± 15.76	315.694 ± 15.42	213.968 ± 10.34
Propionic Acid	3.580 ± 0.30	3.130 ± 0.06	3.389 ± 0.02	3.290 ± 0.19	4.066 ± 0.11	2.674 ± 0.11
Iso-Butyric Acid	2.736 ± 0.28	2.822 ± 0.15	2.779 ± 0.12	2.419 ± 0.12	3.375 ± 0.07	3.076 ± 0.16
Butyric Acid	1.296 ± 0.08	1.125 ± 0.07	1.127 ± 0.05	1.098 ± 0.05	1.101 ± 0.01	1.061 ± 0.03
Iso-Valeric Acid	3.219 ± 0.11	3.531 ± 0.05	3.710 ± 0.13	2.864 ± 0.00	2.899 ± 0.04	2.714 ± 0.01
Valeric Acid	1.224 ± 0.00	1.229 ± 0.00	1.218 ± 0.00	1.223 ± 0.00	1.231 ± 0.00	1.210 ± 0.00
Hexanoic Acid	2.826 ± 0.06	2.582 ± 0.19	2.785 ± 0.11	2.731 ± 0.27	2.635 ± 0.05	2.639 ± 0.10
Octanoic Acid	3.940 ± 0.10	3.763 ± 0.16	3.911 ± 0.13	3.872 ± 0.23	3.732 ± 0.05	3.792 ± 0.10
Decanoic Acid	2.434 ± 0.03	2.396 ± 0.02	2.456 ± 0.01	2.408 ± 0.02	2.396 ± 0.01	2.382 ± 0.00
Total	**253.875**	**281.684**	**287.745**	**319.266**	**337.129**	**233.516**

Nd: not detected

* Refer to **Table 4.4** for a description of the treatments

Table 4.23 Esters, higher alcohols and volatile fatty acids (mg/L) measured in 2009 in Pinotage (sequential inoculation) after MLF with the selected combinations of mixed LAB. Concentrations represent the average of triplicate treatment repeats, each analysed in triplicate by GC-FID. Standard deviations are shown (95% confidence interval).

Treatments *	before MLF	1	2	3	4	5
Esters						
Ethyl Butyrate	0.884 ± 0.00	0.848 ± 0.00	0.847 ± 0.00	0.845 ± 0.00	0.842 ± 0.00	0.849 ± 0.00
Ethyl Hexanoate	1.518 ± 0.00	1.422 ± 0.00	1.429 ± 0.00	1.425 ± 0.00	1.426 ± 0.00	1.427 ± 0.00
Ethyl Lactate	9.774 ± 0.00	47.896 ± 1.00	47.981 ± 0.89	49.102 ± 0.70	27.755 ± 0.62	57.440 ± 0.92
Ethyl Caprylate	0.350 ± 0.00	0.276 ± 0.00	0.284 ± 0.00	0.279 ± 0.00	0.282 ± 0.00	0.290 ± 0.01
Ethyl Caprate	0.276 ± 0.00	0.255 ± 0.00	0.256 ± 0.00	0.257 ± 0.00	0.254 ± 0.00	0.256 ± 0.00
Diethyl Succinate	1.294 ± 0.00	0.885 ± 0.02	0.726 ± 0.01	0.740 ± 0.00	0.647 ± 0.00	0.779 ± 0.01
Ethyl Acetate	56.892 ± 0.00	33.560 ± 0.77	32.128 ± 0.97	33.063 ± 1.41	30.861 ± 3.87	32.628 ± 0.89
Isoamyl Acetate	2.891 ± 0.00	1.436 ± 0.00	1.436 ± 0.00	1.434 ± 0.00	1.444 ± 0.00	1.448 ± 0.00
Hexyl Acetate	0.938 ± 0.00	0.978 ± 0.02	0.971 ± 0.04	1.031 ± 0.06	1.020 ± 0.09	1.016 ± 0.04
2-Phenylethyl Acetate	1.268 ± 0.00	1.156 ± 0.00	1.157 ± 0.00	1.158 ± 0.00	1.157 ± 0.00	1.160 ± 0.00
Total	**76.085**	**88.712**	**87.215**	**89.334**	**65.688**	**97.293**
Alcohols						
Methanol	40.690 ± 0.00	38.140 ± 1.09	36.159 ± 0.45	41.565 ± 3.17	37.051 ± 2.62	37.966 ± 2.49
Propanol	83.196 ± 0.00	48.641 ± 0.72	44.769 ± 0.09	46.227 ± 0.01	46.439 ± 1.11	47.289 ± 1.05
Isobutanol	32.077 ± 0.00	23.712 ± 0.33	23.167 ± 0.34	22.862 ± 0.26	22.518 ± 0.52	23.790 ± 0.25
Butanol	1.534 ± 0.00	1.140 ± 0.01	1.133 ± 0.01	1.118 ± 0.01	1.096 ± 0.00	1.151 ± 0.01
Isoamyl alcohol	207.484 ± 0.00	145.480 ± 1.00	145.890 ± 0.60	146.645 ± 1.23	145.838 ± 0.02	151.336 ± 0.51
Hexanol	2.781 ± 0.00	2.144 ± 0.00	2.146 ± 0.00	2.154 ± 0.00	2.139 ± 0.00	2.163 ± 0.01
2-Phenyl Ethanol	40.147 ± 0.00	22.582 ± 0.17	22.666 ± 0.21	23.087 ± 0.06	23.204 ± 0.30	23.915 ± 0.27
Total	**407.909**	**281.839**	**275.930**	**283.658**	**278.285**	**287.610**
Volatile Fatty Acids						
Acetic acid	175.221 ± 0.00	250.108 ± 6.30	236.368 ± 3.42	237.487 ± 3.93	244.739 ± 4.71	261.151 ± 2.99
Propionic Acid	4.678 ± 0.00	3.769 ± 0.17	3.847 ± 0.13	3.947 ± 0.32	4.019 ± 0.26	4.333 ± 0.04
Iso-Butyric Acid	2.440 ± 0.00	2.588 ± 0.02	2.547 ± 0.07	2.659 ± 0.02	2.361 ± 0.02	2.557 ± 0.07
Butyric Acid	1.176 ± 0.00	1.039 ± 0.01	1.039 ± 0.01	1.039 ± 0.01	1.071 ± 0.02	1.084 ± 0.00
Iso-Valeric Acid	2.691 ± 0.00	3.201 ± 0.00	3.254 ± 0.01	3.307 ± 0.04	3.032 ± 0.06	3.009 ± 0.00
Valeric Acid	1.311 ± 0.00	1.237 ± 0.00	1.237 ± 0.00	1.242 ± 0.00	1.243 ± 0.00	1.247 ± 0.00
Hexanoic Acid	2.774 ± 0.00	2.573 ± 0.01	2.573 ± 0.02	2.590 ± 0.01	2.581 ± 0.01	2.622 ± 0.01
Octanoic Acid	3.937 ± 0.00	3.968 ± 0.02	3.988 ± 0.01	3.989 ± 0.02	4.007 ± 0.01	4.046 ± 0.03
Decanoic Acid	2.489 ± 0.00	2.463 ± 0.01	2.471 ± 0.01	2.478 ± 0.00	2.471 ± 0.01	2.490 ± 0.01
Total	**196.717**	**270.946**	**257.324**	**258.738**	**265.524**	**282.539**

Nd: not detected

* Refer to **Table 4.4** for a description of the treatments

treatments (**Figure 4.16**). This could potentially result in decreased fruitiness in sequential inoculated wines. These preliminary results regarding the effect of inoculation timing on the volatile aroma profile were only investigated in Pinotage and over one vintage and should be further investigated in other cultivars and over a number of vintages.

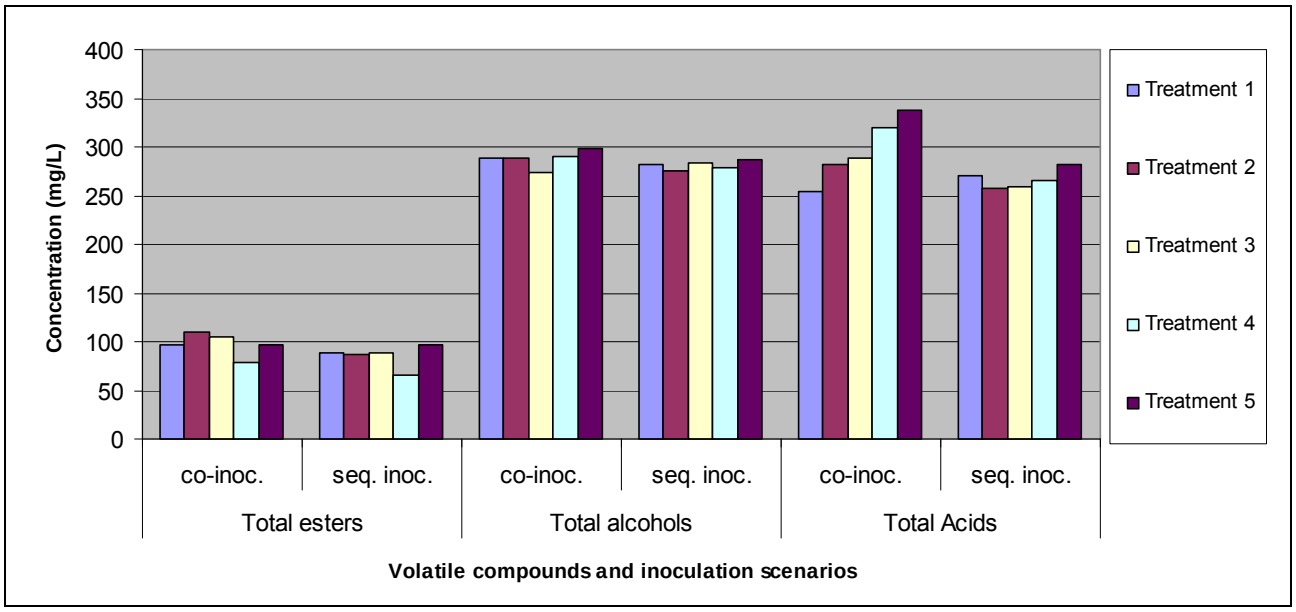

Figure 4.16 The total concentrations (mg/L) of esters, alcohols and volatile fatty acids produced by the MLF treatments (refer to **Table 4.4** for treatment descriptions) in Pinotage 2009 in the two inoculation scenarios (co-inoc: co-inoculation; seq. inoc: sequential inoculation). Each value represents the total of the averages of triplicate treatments. The RSD is less than 10% between fermentation repeats.

In the Cabernet Sauvignon co-inoculated wines (**Table 4.24**), the highest total ester concentration was produced by two of the mixed cultures, treatments 2 and 3, similar to treatment 5. Similar total ester concentrations were observed for treatments 1 and 6. The MLF treatments did not seem to differ in their ability to alter the concentrations of ethyl butyrate, ethyl hexanoate, ethyl caprate, ethyl caprylate, hexyl acetate and 2-phenylethyl acetate. Differences in the treatments were observed for the esters ethyl lactate, diethyl succinate, ethyl acetate and isoamyl acetate. The highest ethyl lactate concentrations were produced by treatments 2 and 3, similar to the production by the commercial culture, treatment 5. Of the three mixed cultures (treatments 2, 3 and 4), treatment 4 produced the lowest ethyl lactate concentration, a trend observed in all four cultivars over both vintages. Quantitatively, treatments 1 and 6 produced the lowest concentrations. The diethyl succinate concentrations were similar for treatment 1 and the three mixed cultures (treatments 2, 3 and 4). The commercial culture (treatment 5) produced the highest diethyl succinate concentrations. A tendency also observed in the Pinotage and Chardonnay co-inoculated wines, the ML01 yeast (treatment 6) were responsible for the lowest concentrations. Ethyl acetate and isoamyl acetate concentrations were the highest in the treatment that had not completed MLF, treatment 1, while similar concentrations were produced by the three mixed cultures (treatments 2, 3 and 4) as well as by treatments 5 and 6.

Similar total ester concentrations were produced by two of the mixed cultures (treatments 1 and 2) and the malolactic yeast ML01 (treatment 5) in the Chardonnay co-inoculated wines (**Table 4.25**). Slightly lower levels were observed for treatment 4 (commercial culture), while the lowest total ester production was observed for treatment 3, the third mixed culture with *O. oeni* E53. Similar concentrations of the ethyl esters (excluding ethyl lactate) and acetate esters were produced by the commercial culture (treatment 4) and the three mixed cultures (treatment 1, 2 and 3), while treatment 5, the malolactic yeast, resulted in slightly higher concentrations of these esters at the completion of MLF. Ethyl lactate concentrations were similar for treatments 1 and 2 and slightly higher than the level observed for treatment 4. As evident in all three cultivars, treatment 3 produced the lowest ethyl lactate concentration, with only a slightly higher concentration associated with fermentation by the malolactic yeast.

4.3.2.2.2 Alcohols

In comparing the total alcohol concentrations in co-inoculated and sequentially inoculated Pinotage wines, it seems that the timing of inoculation has very little or no effect on the higher alcohol production (**Figure 4.16**).

The methanol, butanol, 2-phenyl ethanol, isoamyl alcohol and hexanol concentrations were similar in the co-inoculated Pinotage wines, with only slight differences observed in propanol and isobutanol concentrations. Propanol concentrations were similar for the spontaneous fermentation (treatment 1), one of the mixed cultures (treatment 4) and the malolactic starter culture (treatment 5). The other two mixed cultures, treatments 2 and 3, produced similar concentrations of propanol, while the lowest concentration was observed for treatment 6. Contrary to this, treatment 6 had the highest isobutanol concentration, compared to similar concentrations for treatments 1 to 5. Similar methanol, propanol, isobutanol, butanol and 2-phenyl ethanol levels were associated with the malolactic treatments in the Pinotage sequentially inoculated wines, while similar hexanol and isoamyl alcohol concentrations were evident for all the treatments.

In the Cabernet Sauvignon co-inoculated wines, similar levels of propanol, butanol and isoamyl alcohol was observed in all the treatments, with the exception of treatment 6, which had lower levels of these alcohols. Similarly, methanol, isobutanol and hexanol concentrations were comparable in treatments 1 to 5, while higher levels were produced by treatment 6. The production of 2-phenyl ethanol was inconsistent between the treatments, with similar levels in treatments 1, 3, 4, 6 and treatments 2, 5. In the Chardonnay co-inoculated wines, similar levels of propanol, isobutanol and isoamyl alcohol was observed in all the treatments, with the exception of treatment 5, which had lower levels of these alcohols. The concentration of 2-phenyl ethanol was comparable in treatments 1 to 4, while higher levels were produced by treatment 5. Similar concentrations of butanol and hexanol were observed for all the treatments.

Table 4.24 Esters, higher alcohols and volatile fatty acids (mg/L) measured in 2009 in Cabernet Sauvignon after MLF with the selected combinations of mixed LAB. Concentrations represent the average of triplicate treatment repeats, each analysed in triplicate by GC-FID. Standard deviations are shown (95% confidence interval).

Treatments *	1	2	3	4	5	6
Esters						
Ethyl Butyrate	0.821 ± 0.03	0.824 ± 0.01	0.819 ± 0.01	0.823 ± 0.02	0.816 ± 0.01	0.781 ± 0.01
Ethyl Hexanoate	1.323 ± 0.01	1.312 ± 0.01	1.328 ± 0.01	1.316 ± 0.01	1.312 ± 0.01	1.313 ± 0.01
Ethyl Lactate	22.678 ± 1.05	78.755 ± 0.37	69.614 ± 4.19	41.912 ± 0.59	77.329 ± 7.32	34.398 ± 4.05
Ethyl Caprylate	0.160 ± 0.01	0.167 ± 0.00	0.172 ± 0.00	0.170 ± 0.01	0.169 ± 0.01	0.166 ± 0.00
Ethyl Caprate	0.247 ± 0.00	0.247 ± 0.00	0.247 ± 0.00	0.245 ± 0.00	0.246 ± 0.00	0.245 ± 0.00
Diethyl Succinate	1.433 ± 0.15	1.506 ± 0.07	1.318 ± 0.05	1.335 ± 0.09	1.666 ± 0.03	1.292 ± 0.09
Ethyl Acetate	56.501 ± 0.25	49.935 ± 0.55	53.225 ± 2.84	54.206 ± 2.19	42.695 ± 1.10	44.594 ± 1.74
Isoamyl Acetate	1.563 ± 0.03	1.434 ± 0.07	1.460 ± 0.02	1.467 ± 0.05	1.437 ± 0.07	1.336 ± 0.05
Hexyl Acetate	0.966 ± 0.04	1.013 ± 0.08	0.940 ± 0.02	0.932 ± 0.01	0.946 ± 0.02	1.064 ± 0.03
2-Phenylethyl Acetate	1.194 ± 0.01	1.184 ± 0.01	1.189 ± 0.00	1.191 ± 0.00	1.185 ± 0.01	1.194 ± 0.02
Total	**86.886**	**136.377**	**130.312**	**103.359**	**127.801**	**86.383**
Alcohols						
Methanol	73.738 ± 2.54	82.122 ± 3.71	65.379 ± 8.10	71.107 ± 2.25	74.266 ± 4.20	81.486 ± 5.08
Propanol	60.356 ± 0.68	52.691 ± 4.08	53.784 ± 1.01	51.258 ± 0.65	46.356 ± 0.45	36.059 ± 0.51
Isobutanol	39.114 ± 2.38	36.149 ± 1.46	33.821 ± 0.85	34.365 ± 0.49	32.918 ± 2.64	43.660 ± 1.67
Butanol	1.962 ± 0.16	2.460 ± 0.08	2.389 ± 0.08	2.422 ± 0.15	2.485 ± 0.07	1.556 ± 0.06
Isoamyl alcohol	244.779 ± 11.98	252.045 ± 6.33	246.447 ± 2.80	230.550 ± 4.50	242.300 ± 11.70	227.268 ± 8.33
Hexanol	2.444 ± 0.00	2.474 ± 0.01	2.506 ± 0.01	2.485 ± 0.02	2.465 ± 0.02	2.547 ± 0.01
2-Phenyl Ethanol	48.654 ± 3.99	63.445 ± 3.22	55.241 ± 3.95	53.355 ± 0.45	60.691 ± 2.02	54.142 ± 4.25
Total	**471.047**	**491.386**	**459.567**	**445.542**	**461.481**	**446.718**
Volatile Fatty Acids						
Acetic acid	144.912 ± 0.16	161.529 ± 19.13	176.782 ± 15.51	153.464 ± 9.20	130.736 ± 10.58	69.283 ± 9.63
Propionic Acid	3.351 ± 0.32	3.855 ± 0.03	3.600 ± 0.01	3.355 ± 0.28	3.332 ± 0.09	2.742 ± 0.07
Iso-Butyric Acid	2.648 ± 0.05	3.263 ± 0.10	2.954 ± 0.08	2.755 ± 0.18	2.853 ± 0.06	2.634 ± 0.18
Butyric Acid	0.936 ± 0.02	0.945 ± 0.01	0.943 ± 0.01	0.884 ± 0.01	0.905 ± 0.01	0.828 ± 0.01
Iso-Valeric Acid	3.270 ± 0.10	3.362 ± 0.15	3.485 ± 0.07	3.144 ± 0.05	3.066 ± 0.07	2.796 ± 0.02
Valeric Acid	1.239 ± 0.00	1.262 ± 0.01	1.245 ± 0.01	1.234 ± 0.00	1.249 ± 0.01	1.219 ± 0.00
Hexanoic Acid	2.021 ± 0.03	1.972 ± 0.03	2.039 ± 0.02	1.969 ± 0.01	1.963 ± 0.04	1.992 ± 0.01
Octanoic Acid	3.101 ± 0.05	3.079 ± 0.01	3.120 ± 0.02	3.082 ± 0.03	3.082 ± 0.05	3.077 ± 0.01
Decanoic Acid	2.285 ± 0.00	2.273 ± 0.01	2.279 ± 0.01	2.270 ± 0.01	2.245 ± 0.02	2.274 ± 0.01
Total	**163.763**	**181.540**	**196.447**	**172.157**	**149.431**	**86.845**

Nd: not detected

* Refer to **Table 4.4** for a description of the treatments

Results suggest that the higher alcohol production is not so much strain dependant, with no or slight differences between the LAB treatments. The differences that are evident is as a result of MLF with the malolactic yeast.

4.3.2.2.3 Volatile Fatty Acids

The timing of inoculation seems to have only a slight effect on the fatty acid profiles of wines that had undergone MLF. Co-inoculated MLF seems to lead to a slightly higher concentration of total fatty acids compared to sequential inoculation (**Figure 4.16**). There were no clearly discernable trends visible in the Pinotage co-inoculated wines. There were no differences between treatments regarding the production of valeric acid, hexanoic acid, octanoic- or decanoic acid. The levels of propionic acid and iso-butyric acid were similar for treatments 1, 2, 3 and 4, while slightly lower propionic acid and slightly higher iso-butyric levels were associated with treatment 6, the ML01 yeast. Similar concentrations of fatty acids were associated with the spontaneous fermentation (treatment 1) and the three mixed cultures (treatments 2, 3 and 4) in Pinotage sequentially inoculated wines, while a higher total acid concentration was observed for treatment 5. This trend was also observed in the concentrations of propionic acid and hexanoic acid, while there were no noticeable differences between the levels of iso-butyric acid, butyric acid, valeric acid, octanoic- and decanoic acids in the different treatments.

The highest total fatty acid concentrations in Cabernet Sauvignon were associated with the three mixed culture treatments (2, 3 and 4), while treatment 6 had the lowest total acid concentration. Similar acid concentrations for treatments 1 to 5 and the lowest levels for treatment 6 were observed for propionic acid, isobutyric acid, butyric acid and iso-valeric acid. No discernable differences between treatments were observed for valeric acid, hexanoic acid, octanoic- and decanoic acid.

Chardonnay wines had higher total acid concentrations compared to Pinotage and Cabernet Sauvignon. Similar total acid concentrations were observed for treatments 1 to 4. Despite the fact that treatment 5 had the highest concentrations of butyric acid, valeric acid, hexanoic acid, octanoic acid and decanoic acid, it had a lower total acid concentration due to the production of a smaller acetic acid concentration. Similar levels were observed in all the treatments for valeric acid, propionic acid and iso-butyric acid, with the exception of lower levels of propionic acid and iso-butyric acid in treatment 5.

Table 4.25 Esters, higher alcohols and volatile fatty acids (mg/L) measured in 2009 in Chardonnay after MLF with the selected combinations of mixed LAB. Concentrations represent the average of triplicate treatment repeats, each analysed in triplicate by GC-FID. Standard deviations are shown (95% confidence interval).

Treatments *	1	2	3	4	5
Esters					
Ethyl Butyrate	1.079 ± 0.01	1.064 ± 0.01	1.065 ± 0.00	1.061 ± 0.01	1.177 ± 0.05
Ethyl Hexanoate	1.898 ± 0.02	1.885 ± 0.03	1.881 ± 0.02	1.872 ± 0.01	2.174 ± 0.14
Ethyl Lactate	26.626 ± 0.48	27.841 ± 0.66	14.262 ± 0.28	23.748 ± 0.15	18.772 ± 0.56
Ethyl Caprylate	0.649 ± 0.00	0.642 ± 0.02	0.630 ± 0.01	0.634 ± 0.01	0.932 ± 0.01
Ethyl Caprate	0.330 ± 0.00	0.331 ± 0.00	0.323 ± 0.00	0.329 ± 0.00	0.347 ± 0.01
Diethyl Succinate	0.449 ± 0.01	0.458 ± 0.02	0.446 ± 0.01	0.449 ± 0.01	0.396 ± 0.00
Ethyl Acetate	47.167 ± 0.61	44.899 ± 0.56	44.785 ± 0.94	44.856 ± 2.73	52.253 ± 2.62
Isoamyl Acetate	4.050 ± 0.13	3.897 ± 0.15	3.934 ± 0.09	3.940 ± 0.02	5.694 ± 0.08
Hexyl Acetate	1.197 ± 0.01	1.194 ± 0.01	1.188 ± 0.01	1.189 ± 0.01	1.283 ± 0.06
2-Phenylethyl Acetate	1.362 ± 0.00	1.352 ± 0.02	1.337 ± 0.01	1.348 ± 0.00	1.548 ± 0.05
Total	**84.807**	**83.563**	**69.851**	**79.426**	**84.576**
Alcohols					
Methanol	69.437 ± 4.24	74.007 ± 7.81	88.631 ± 2.37	91.531 ± 0.98	73.207 ± 9.21
Propanol	59.043 ± 0.55	60.548 ± 1.46	58.879 ± 1.24	57.731 ± 1.63	49.638 ± 1.21
Isobutanol	12.951 ± 0.11	13.082 ± 0.23	12.816 ± 0.20	12.672 ± 0.32	11.425 ± 0.15
Butanol	0.865 ± 0.00	0.883 ± 0.01	0.857 ± 0.01	0.842 ± 0.01	0.851 ± 0.03
Isoamyl alcohol	102.444 ± 0.44	102.377 ± 1.38	101.637 ± 1.09	101.489 ± 0.25	97.729 ± 1.23
Hexanol	2.273 ± 0.01	2.282 ± 0.01	2.269 ± 0.00	2.260 ± 0.00	2.210 ± 0.00
2-Phenyl Ethanol	13.404 ± 0.19	13.309 ± 0.47	12.925 ± 0.38	13.029 ± 0.14	17.714 ± 1.05
Total	**260.417**	**266.488**	**278.014**	**279.554**	**252.774**
Volatile Fatty Acids					
Acetic acid	196.844 ± 7.18	225.272 ± 11.31	224.588 ± 6.45	202.084 ± 3.63	87.146 ± 2.10
Propionic Acid	3.386 ± 0.16	3.776 ± 0.27	3.264 ± 0.20	3.184 ± 0.12	2.477 ± 0.01
Iso-Butyric Acid	2.166 ± 0.04	2.452 ± 0.17	2.169 ± 0.14	2.121 ± 0.17	1.521 ± 0.03
Butyric Acid	1.424 ± 0.01	1.445 ± 0.02	1.445 ± 0.03	1.443 ± 0.01	1.637 ± 0.07
Iso-Valeric Acid	2.572 ± 0.00	2.577 ± 0.01	2.586 ± 0.04	2.572 ± 0.02	2.586 ± 0.04
Valeric Acid	1.281 ± 0.00	1.299 ± 0.00	1.288 ± 0.00	1.287 ± 0.00	1.311 ± 0.01
Hexanoic Acid	4.303 ± 0.02	4.280 ± 0.04	4.207 ± 0.07	4.218 ± 0.06	5.340 ± 0.33
Octanoic Acid	6.728 ± 0.02	6.670 ± 0.10	6.545 ± 0.11	6.569 ± 0.08	8.103 ± 0.56
Decanoic Acid	3.559 ± 0.06	3.591 ± 0.06	3.500 ± 0.03	3.542 ± 0.03	3.751 ± 0.17
Total	**222.263**	**251.362**	**249.592**	**227.020**	**113.872**

Nd: not detected

* Refer to **Table 4.4** for a description of the treatments

4.3.2.2.4 Carbonyl compounds

Diacetyl is considered one of the most important aroma compounds associated with MLF and is formed as an intermediate of the citric acid metabolism by LAB. Other intermediates in this pathway include 2,3-butanediol, acetoin and 2,3-pentanedione (Bartowsky *et al*., 2002). Diacetyl contributes buttery, nutty and butterscotch characters to the wine and can be further reduced to 2,3-butanediol and acetoin and although these compounds also impart a buttery character to the wine, they have higher threshold values and therefore contribute to the buttery aroma to a lesser extent. The aroma threshold for diacetyl in red wine has been determined as 2.8 mg/L, but at concentrations of 1 to 4 mg/L, diacetyl can still add to wine complexity and the buttery aroma (Bartowsky and Henschke, 2004). The results of the GC-MS analysis of wines from the 2009 vintage are listed in **Table 4.26**.

Table 4.26 The carbonyl compounds (mg/L) measured in 2009 in Pinotage, Cabernet Sauvignon and Chardonnay after MLF with the combination of mixed isolates. Concentrations represent the average of triplicate treatment repeats, analysed by GC-MS. Standard deviations are not shown.

Treatment number *	Carbonyl compounds		
	Diacetyl	Acetoin	2,3-Pentanedione
Pinotage co-inoculation			
1	1.625	1.750	nd
2	1.640	1.725	nd
3	1.615	1.510	nd
4	1.620	1.530	nd
5	nd	1.217	nd
6	nd	8.575	nd
Pinotage sequential inoculation			
1	1.633	2.353	nd
2	1.627	3.237	nd
3	1.635	3.340	nd
4	1.650	14.193	nd
5	nd	1.137	nd
Cabernet Sauvignon co-inoculation			
1	nd	1.415	nd
2	1.633	5.097	nd
3	1.625	4.027	nd
4	1.640	6.293	nd
5	nd	3.377	nd
6	nd	3.520	nd
Chardonnay co-inoculation			
1	nd	8.597	nd
2	nd	7.073	nd
3	nd	15.020	nd
4	nd	5.545	nd
5	nd	5.030	nd

* See **Table 4.4** for treatment descriptions
nd: not detected

Some of the wines contained diacetyl concentrations at levels that could add to wine aroma complexity and may impart buttery nuances despite being under the sensory threshold value. The odour threshold for acetoin is 150 mg/L and none of the treatments produced concentrations nearing this. Similar concentrations of diacetyl were produced in the red wine cultivars, while no detectable diacetyl concentrations were produced in Chardonnay. This could be due to the reduction of diacetyl to acetoin and also due to the fact that red wine favours the formation of diacetyl compared to white wine (Bartowsky *et al*., 2002). This theory is supported by the higher concentrations of acetoin in Chardonnay compared to acetoin levels in Pinotage and Cabernet Sauvignon. The mixed culture with *O. oeni* E53 and *Lb. plantarum* 14.1, consistently had higher concentrations of acetoin, except in the Pinotage co-inoculation. There were no detectable concentrations of 2,3-pentanedione in any of the samples. The commercial malolactic starter culture as well the ML01 yeast (treatments 5 and/or 6), had no detectable levels of diacetyl. Sequential inoculation seems to produce higher concentrations of acetoin, despite similar diacetyl concentrations being present.

Results obtained in this study confirm the profound effect that MLF has on the wine aroma profile, as well as the effect that different LAB strains have on volatile aroma compound production. Information on the characterised aroma profiles associated with the different bacteria cultures, inoculation times and cultivars evaluated in this study, make it possible to produce a certain style of wine by selecting a specific bacteria culture for MLF. The bacteria cultures evaluated in this study were able to influence the fruity character, as well as mouthfeel of the wines. The *O. oeni* strains in this study seem to have a greater effect on the ester profiles of the wines, while higher alcohol and volatile fatty acid production seemed to be influenced by the *Lb. plantarum* strain present in the mixed culture. It is clear that the cultures evaluated during the course of this study are able to make positive and pronounced contributions to the wine aroma profile.

4.3.3 DATA ANALYSIS

Spectral data obtained by FT-IR and chemical data obtained by FT-IR, GC-FID and GC-MS, were imported into *The Unscrambler* software for the purpose of PCA. This is a technique used to reduce complex dimensional data matrices, with the retention of maximum variability (Naes *et al*., 2002). The projection of the samples in a multi-dimensional space allows for the identification of the main directions of variance, depicted by a principal component (PC). This allows for the interpretation of relationships between various samples in the score plot defined by the PC's and elucidate the relationship between variables and objects in the loadings plot. PCA allows for possible sample groupings to be identified when samples with similar (sharing high loadings for some compounds in the loadings plot) aroma compositions cluster together. Similarly, samples which differ in aroma composition can be discriminated. In addition, variables mostly responsible for differences between samples could be identified. Numbers used in the PCA score plots refer to treatment numbers (refer to **Table 4.3** and **Table 4.4** for treatment descriptions).

4.3.3.1 2008

PCA was used to summarise the information contained in the multivariate data set. PCA's were performed with the samples and variables including the spectral data, GC-FID data, major wine parameters determined with FT-IR spectroscopy and GC-MS data. The FT-IR spectrum serves as a chemical 'fingerprint' and could contain additional hidden information contributing to the separation of the samples along the principal component. Variables with higher loadings (variables that are further from the origin on the loadings plot), are generally the important variables explaining the separation or distribution along a specific principal component. These variables with higher loadings contribute to the structure of the dataset (Malherbe, 2007).

PCA was performed on the Pinotage wines, which included the 11 MLF treatments and all wavenumbers from the spectral dataset (excluding the water absorbance regions 1543-1916 cm^{-1} and 2970-3625 cm^{-1}) (**Figure 4.17**). It was possible to separate the treatment samples for before MLF (before MLF) and the spontaneous fermentation treatment (11) that did not undergo MLF, from the mixed culture treatments. Supplementary PCA was performed on these samples using the GC-FID generated data on the various esters (**Figure 4.18**). On the score plot, separation along PC2 was associated with discrimination of treatments 3, 6 and 9 from the other MLF treatments. High loadings for ethyl lactate was negatively correlated with treatments 3, 6 and 9. These three treatments consist of *O. oeni* E53 as part of the mixed culture.

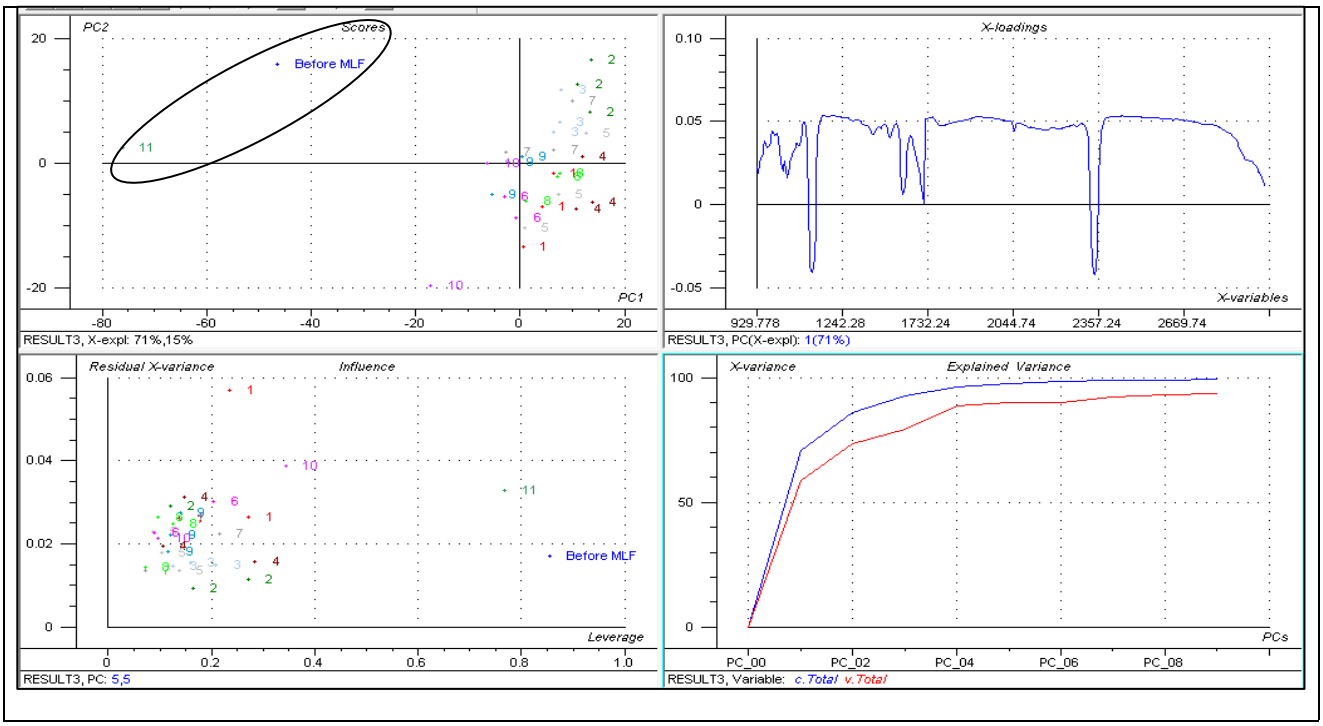

Figure 4.17 PCA score plot (PC1 vs. PC2) of the MLF treatment samples in Pinotage in 2008. PC1 explains 71% of the variance between the samples. Two groups were separated based on discrimination by the spectral data.

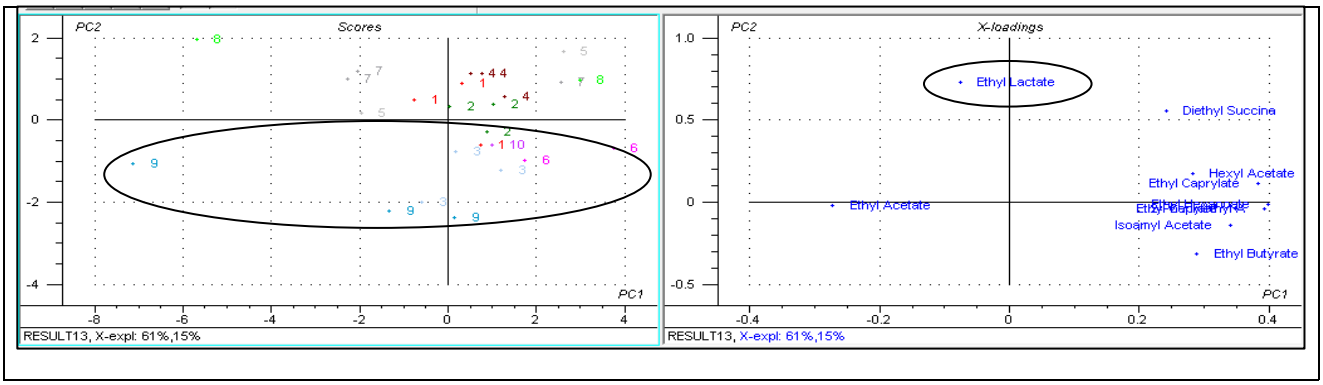

Figure 4.18 PCA score plot (PC1 vs. PC2) of the MLF treatment samples in Pinotage in 2008. Discrimination of the treatments are based on the ester profile of the samples and PC1 explains 61% of the variance in the data structure.

PCA was also performed on these samples using the GC-FID generated data on the various higher alcohols (**Figures 4.19**) and fatty acids (**Figure 4.20**). On the score plot for the higher alcohols, separation along PC1 was associated with discrimination of treatments 7, 8 and 9 from the other MLF treatments. High loadings for 2-phenyl ethanol, isoamyl alcohol, iso-butanol, butanol, propanol and methanol were correlated with treatments 7, 8 and 9. Separation along PC1 was associated with discrimination between the remaining treatments with high loadings for hexanol.

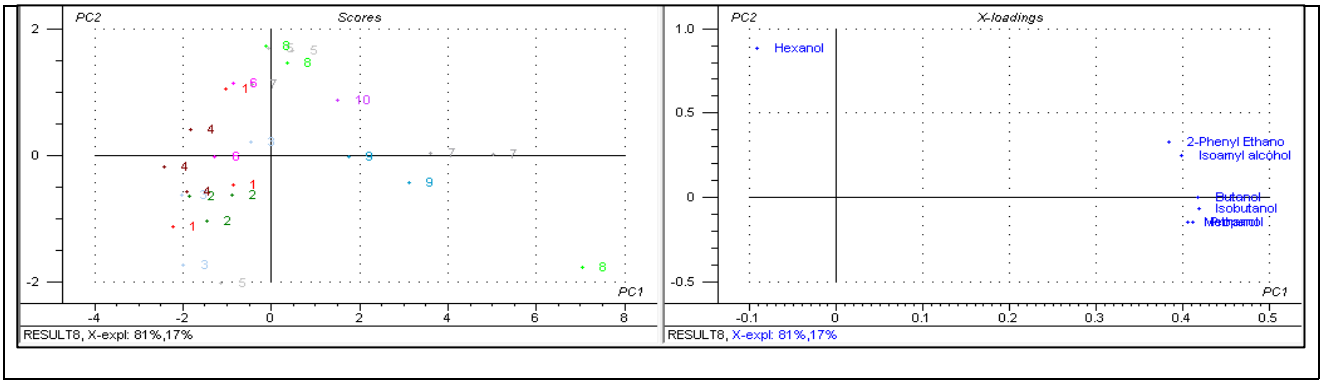

Figure 4.19 PCA score plot (PC1 vs. PC2) of the MLF treatment samples in Pinotage in 2008. Discrimination of the treatments is based on the higher alcohol profile of the samples and PC1 explains 81% of the variance in the data structure.

On the score plot in **Figure 4.20,** separation along PC1 was associated with discrimination of treatments 7, 8 and 9 from the other MLF treatments. High loadings for isovaleric acid, butyric acid, iso-butyric acid and to a lesser extent acetic acid and propionic acid, were correlated with treatments 7, 8 and 9. The low loadings for valeric acid implies that this compound does not contribute to the data structure. Separation along the first PC also demonstrated high loadings for hexanoic acid and decanoic- and octanoic acid which were correlated with the remaining treatments.

Treatment 7, 8 and 9 contain *Lb. plantarum* 107 as part of the mixed LAB culture. It seems as if the *O. oeni* strain selected for MLF has a more pronounced effect on the ester profile, compared to the more pronounced influence of the *Lb. plantarum* strain on the volatile acid and higher alcohol profiles of the Pinotage wines.

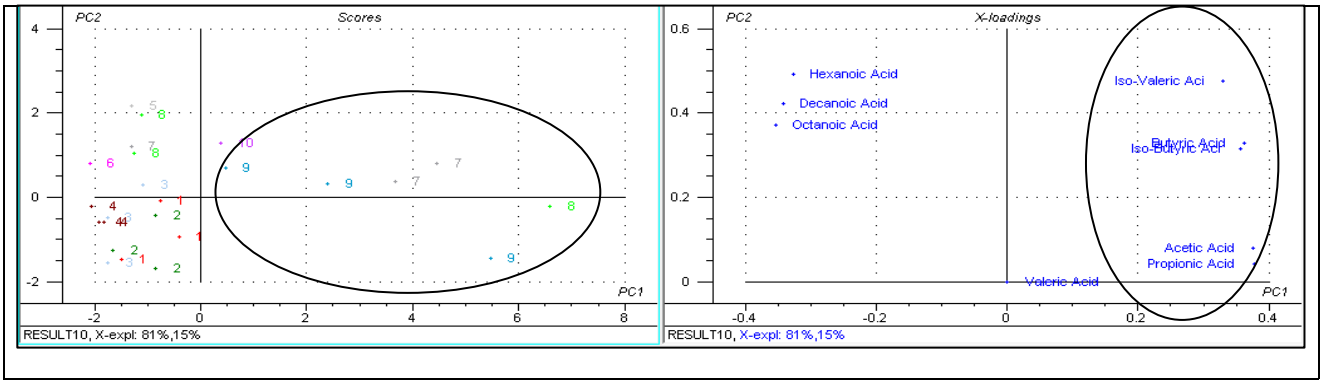

Figure 4.20 PCA score plot (PC1 vs. PC2) of the MLF treatment samples in Pinotage in 2008. Discrimination of the treatments is based on the volatile acid profile of the samples and PC1 explains 81% of the variance in the data structure.

PCA was performed on the Cabernet Sauvignon wines which included the 11 MLF treatments, all wave numbers from the spectral dataset (excluding the water absorbance regions 1543-1916 cm^{-1} and 2970-3625 cm^{-1}) and GC-FID data (**Figure 4.21**).

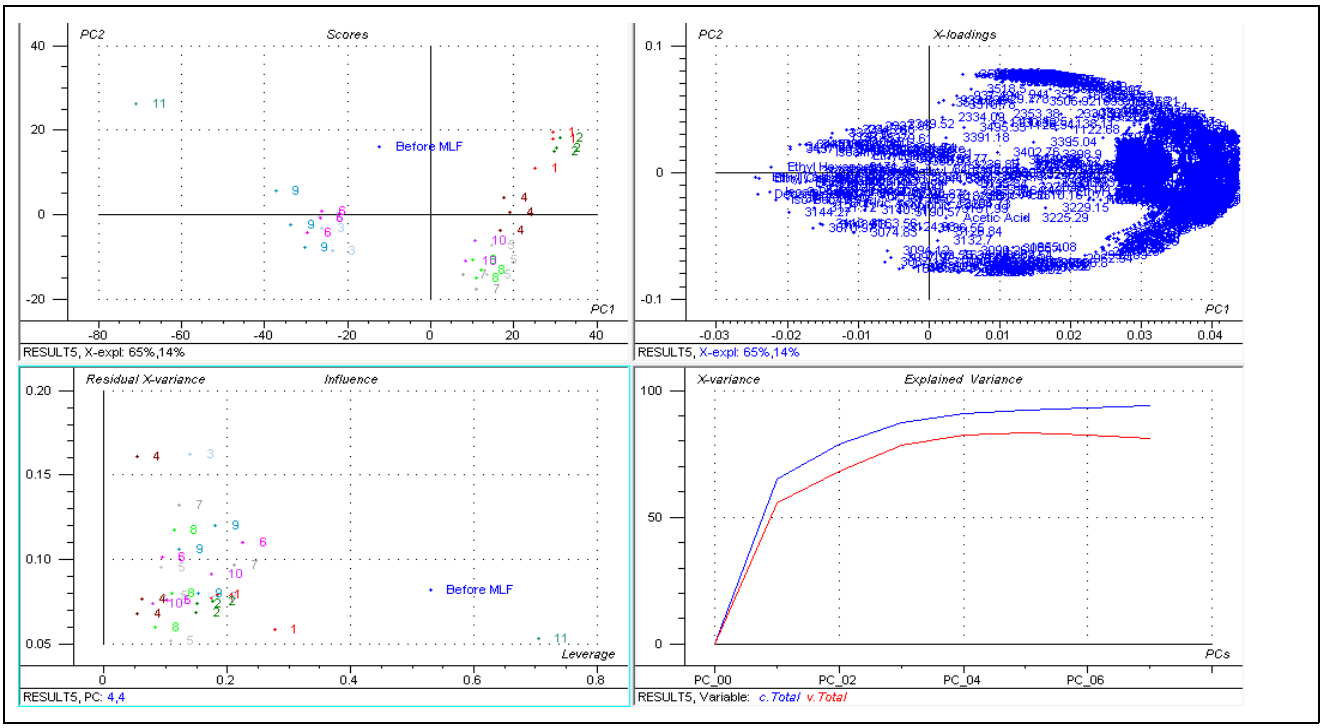

Figure 4.21 PCA score plot (PC1 vs. PC2) of the MLF treatment samples in Cabernet Sauvignon in 2008. PC1 explains 65% of the variance between the samples. The treatments separated along PC1 and discrimination is based on the spectral data and GC-FID data.

On the score plot it can be seen that the treatments separated along PC1 and two groupings are visible as well as separation of treatment 11 and the before MLF sample. The samples that had not undergone MLF separated from the remaining treatments. Treatments 3, 6 and 9 separated towards the left, while the remaining treatments separated towards the right. The same groupings and separation were observed in PCA using only the spectral data. Supplementary PCA was performed on these samples using only the GC-FID data (**Figure 4.22**), since several features concerning the data structure might have been lost due to the domination of the spectral variables. Similar to results obtained in the Pinotage, treatments 3, 6 and 9 were negatively correlated with ethyl lactate, while high loadings for the remaining volatile compounds, excluding valeric acid and hexyl acetate that had low loadings, were responsible for separation along PC1. It is clear that *O. oeni* E53 in treatments 3, 6 and 9 could have a pronounced effect on the aroma profile of the wine.

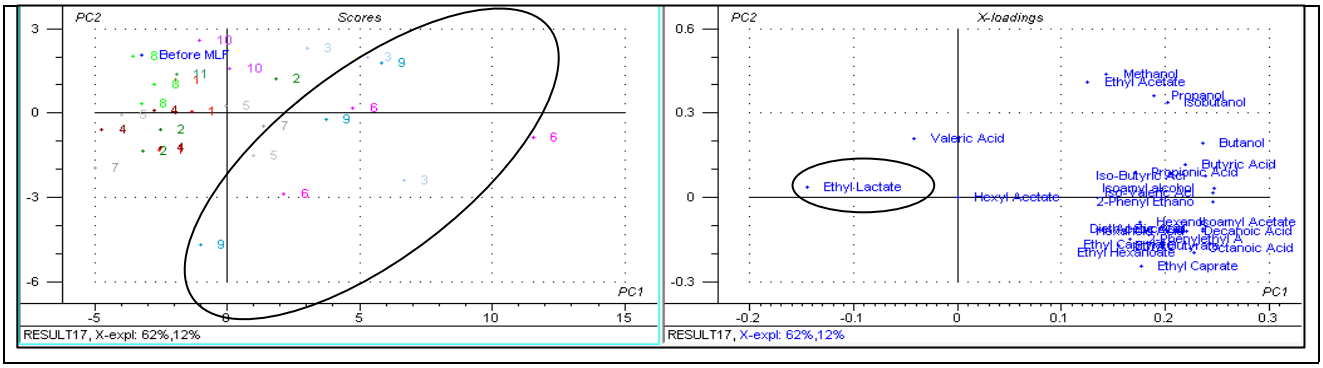

Figure 4.22 PCA score plot (PC1 vs. PC2) of the MLF treatment samples in Cabernet Sauvignon in 2008. PC1 explains 62% of the variance in the data structure. The treatments separated along PC1 and discrimination is based on GC-FID data.

Results of a PCA performed on the treatment samples in Shiraz wine using only the spectral data (excluding the water absorbance regions 1543-1716 cm^{-1} and 2970-3625 cm^{-1}), can be seen in **Figure 4.23**. No clear discriminations were observed between the MLF treatments, with the exception of treatments 1 and 11.

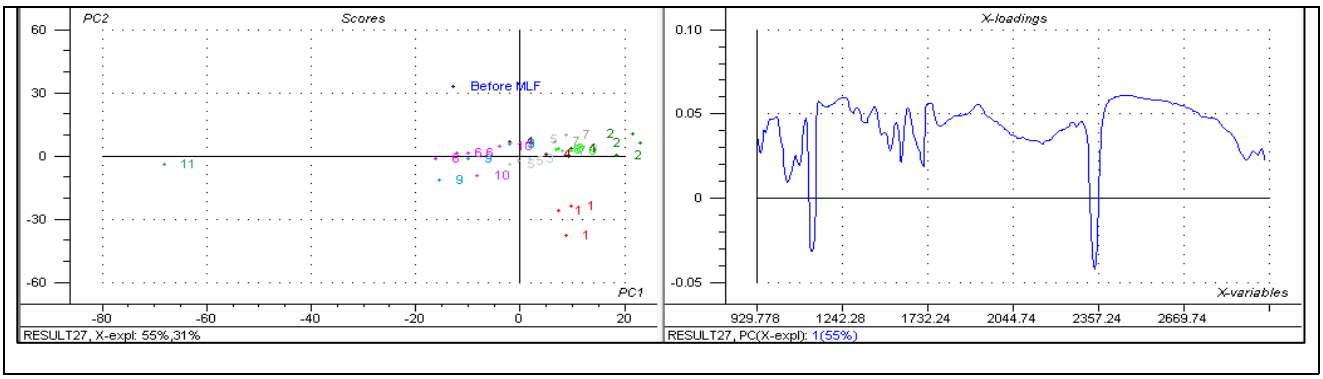

Figure 4.23 PCA score plot (PC1 vs. PC2) generated with the spectral data of the MLF treatment samples in Shiraz in 2008. PC1 explains 55% of the variance in the data structure. No clear discrimination were observed.

4.3.3.2 2009

The differences due to the timing of inoculation, as well as variability between treatments, were investigated in datasets from wines in the 2009 vintage. PCA was performed on the co-inoculated samples and the sequentially inoculated samples, but also on the combined Pinotage wine samples to investigate the differences between co-inoculation and sequential inoculation and also if these inoculation scenarios lead to different wine aroma profiles.

PCA was performed on Pinotage which included all the MLF treatment samples, all wave numbers (excluding the water absorbance regions 1543-1716 cm^{-1} and 2970-3625 cm^{-1}), GC-FID and GC-MS data (**Figure 4.24**). Additional category variable information in the dataset made it possible to observe that co-inoculation and sequential inoculation of the LAB treatments could be distinguished from each other and separated along PC1, while ML01 (malolactic yeast) formed a separate grouping despite being a co-inoculated treatment.

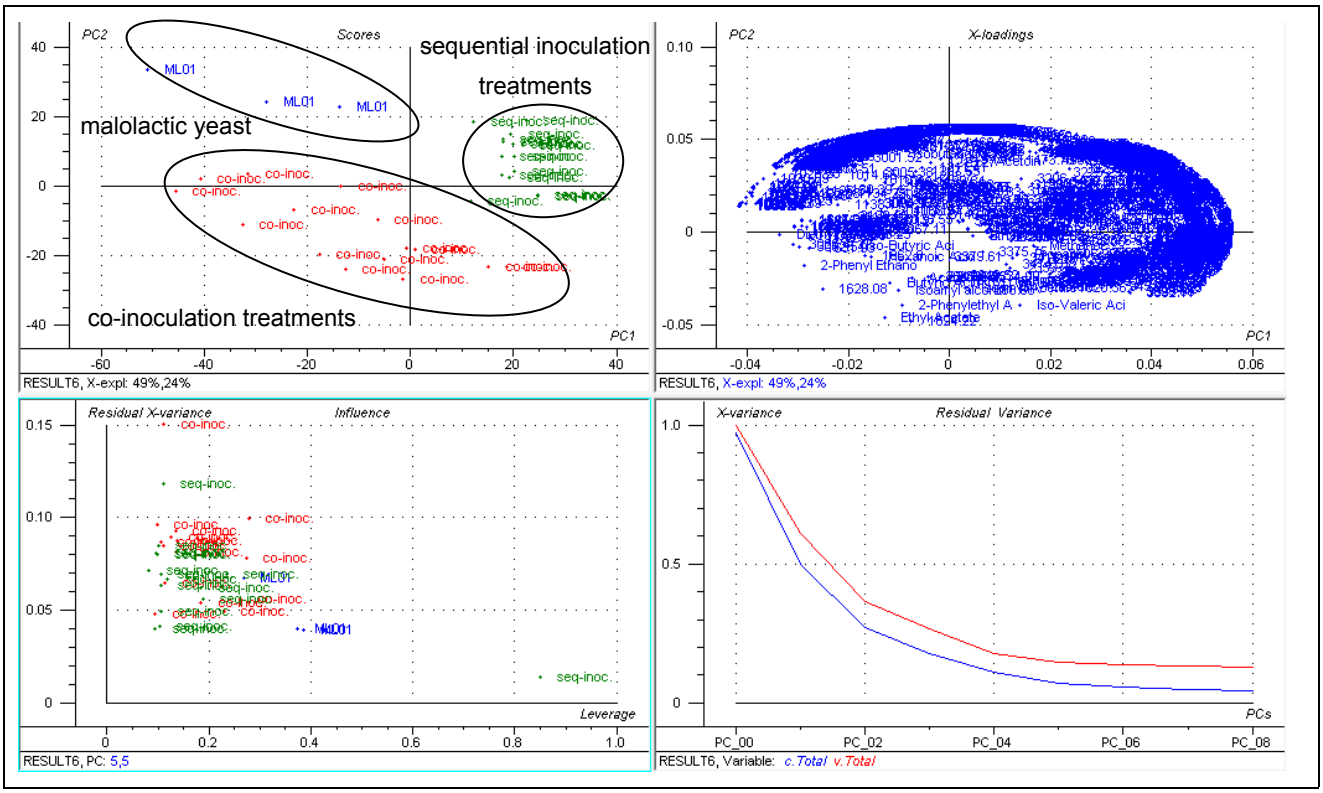

Figure 4.24 Score plot (PC1 vs. PC2) of all the 2009 Pinotage samples. PC1 explains 49% of the variance between the samples. Discrimination between co-inoculation (red) and sequential inoculation (green) and the co-inoculated ML01 treatments (blue) were observed.

Supplementary PCA was performed on the samples using only the GC-FID data (**Figure 4.25**) to investigate the possible differences in the aroma profiles due to a specific inoculation scenario. On the score plot, separation along PC1 was associated with discrimination between the co-inoculated, sequentially inoculated and ML01 samples. High loadings for 2-phenyl ethanol, 2-phenylethyl acetate, isoamyl alcohol, diethyl succinate, isobutyric acid, hexanol and isobutanol were correlated with ML01. Separation along the first PC also demonstrated high loadings for

valeric acid, butyric acid, decanoic-, propionic- and octanoic acid, hexanoic acid, butanol, propanol, ethyl acetate, ethyl caprylate, ethyl butyrate, ethyl hexanoate and isoamyl acetate which were correlated with sequential inoculation and co-inoculation. Low loadings for iso-valeric acid, acetic acid, methanol, ethyl caprate, hexyl acetate and ethyl lactate had little effect on the data structure and as a result did not account for much variability between the samples. Differences in ethyl lactate concentrations, one of the most important esters associated with MLF, seem to be more strain dependant than influenced by the timing of inoculation.

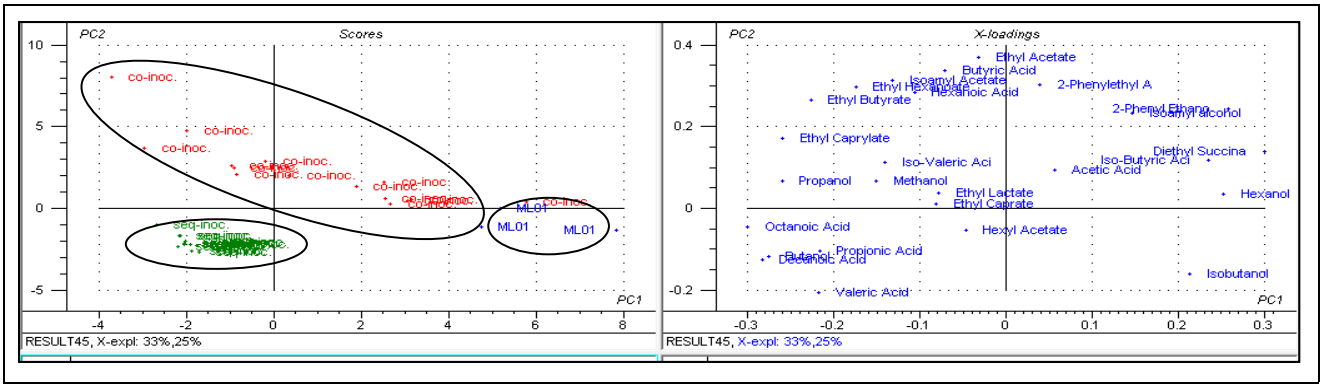

Figure 4.25 Score plot (PC1 vs. PC2) of all the 2009 Pinotage samples. PC1 explains 33% of the variance between the samples. Discrimination between co-inoculation (red) and sequential inoculation (green) and the co-inoculated ML01 treatments (blue) based on GC-FID were observed.

Additional PCA's were performed on the samples using the different groups of volatile compounds as determined by GC-FID and GC-MS analysis (**Figure 4.26**). Esters, higher alcohols, volatile fatty acids and carbonyl compounds were used to perform PCA and investigate the differences in the production of these compounds associated with the different inoculation times. High loadings for diethyl succinate was correlated with ML01, while low loadings of hexyl acetate, ethyl caprate and ethyl lactate had smaller roles to play in the structure of the data set. High loadings for the remaining esters seem to be correlated with co-inoculation. It is possible that the ester profile of a wine could be more affected and influenced during co-inoculation, compared to sequential inoculation where precursors might already have been utilised by the yeast during AF.

High loadings for methanol, propanol and butanol was correlated with sequential inoculation, while high loadings for the remainder of the higher alcohols was correlated with ML01 and the separation of co-inoculation along PC1. High loadings for isobutyric acid was correlated with ML01; hexanoic acid, acetic acid and butyric acid with co-inoculation and octanoic, iso-valeric acid, propionic acid and valeric acid with sequential inoculation.

The production of diacetyl and acetoin seem to be strain dependant, rather than being influenced by the timing of inoculation. Due to the fact that there were no detectable amounts of 2,3-pentanedione in any of the samples, this variable had a low loading and had no effect on the data structure, while ML01 showed a negative correlation with diacetyl.

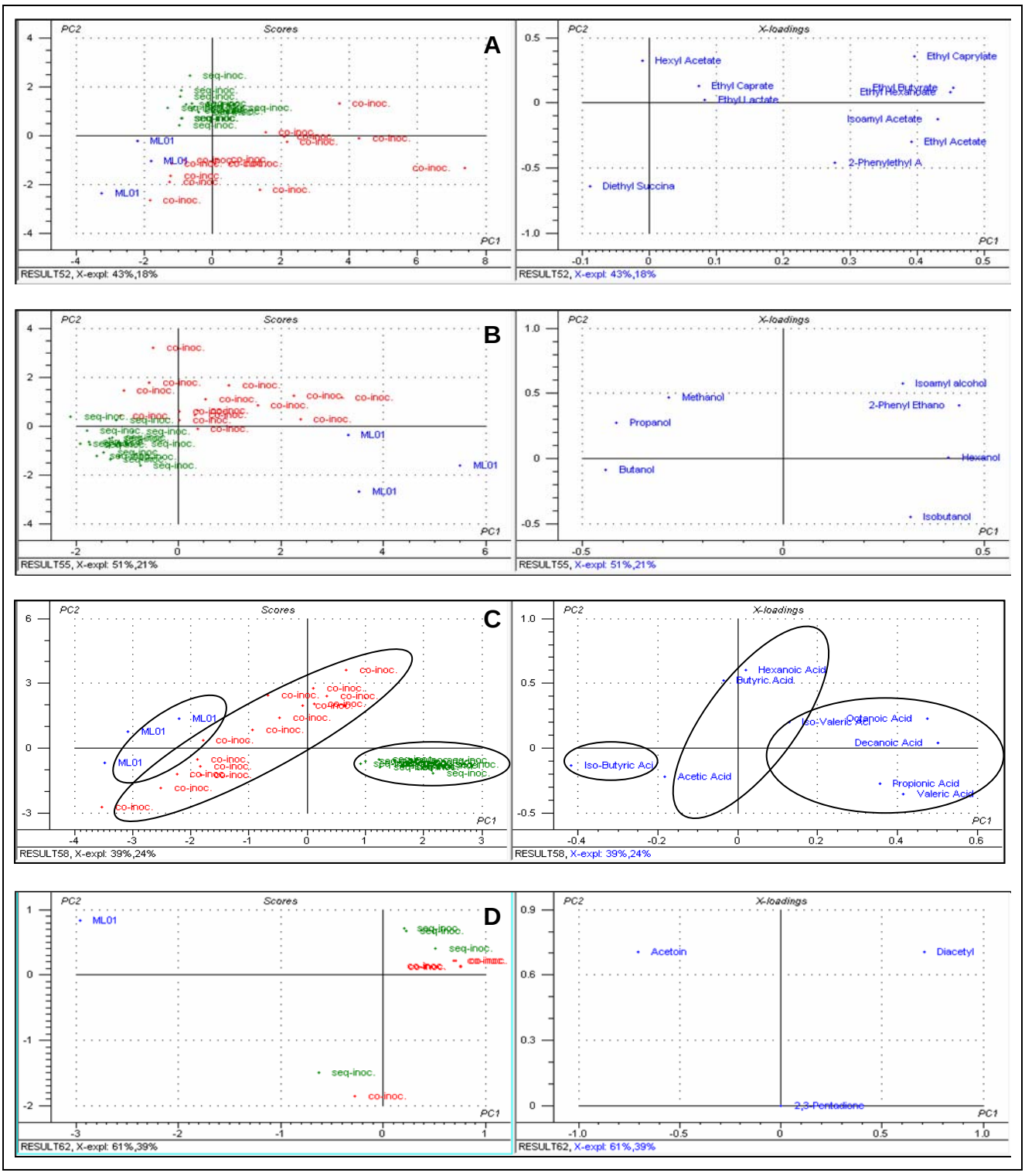

Figure 4.26 Score plots (PC1 vs. PC2) of all the 2009 Pinotage samples. PC1 explains 43%, 51%, 39% and 61% of the variance between the samples in A, B, C and D, respectively. Discrimination between co-inoculation (red) and sequential inoculation (green) and the co-inoculated ML01 treatments (blue) were based on the ester (A), higher alcohols (B), volatile fatty acids (C) and carbonyl compounds (D) measured with GC-FID and GC-MS.

PCA of the individual co-inoculation and sequential inoculation samples showed very few discernable differences between the treatments, with the only discriminations between the treatments evaluated in the sequential inoculation. On the score plot of the PCA with the GC-FID

data and the sequential samples in Pinotage, separation along PC1 was associated with the difference between treatment 5 and the other treatments (**Figure 4.27**). High loadings for iso-valeric acid was negatively correlated with treatment 5, while high loadings for the remainder of the volatile compounds positively correlated with treatment 5. This result suggests that the commercial malolactic starter culture, VP41, produces a different sensorial profile than the mixed LAB cultures evaluated in this study. In a PCA with the esters determined with GC-FID analysis and the sequential inoculation samples, the same result as observed in the previous vintage and other cultivars were once again repeated here. Treatment 4, containing *O. oeni* E53, correlated negatively with ethyl lactate (**Figure 4.28**).

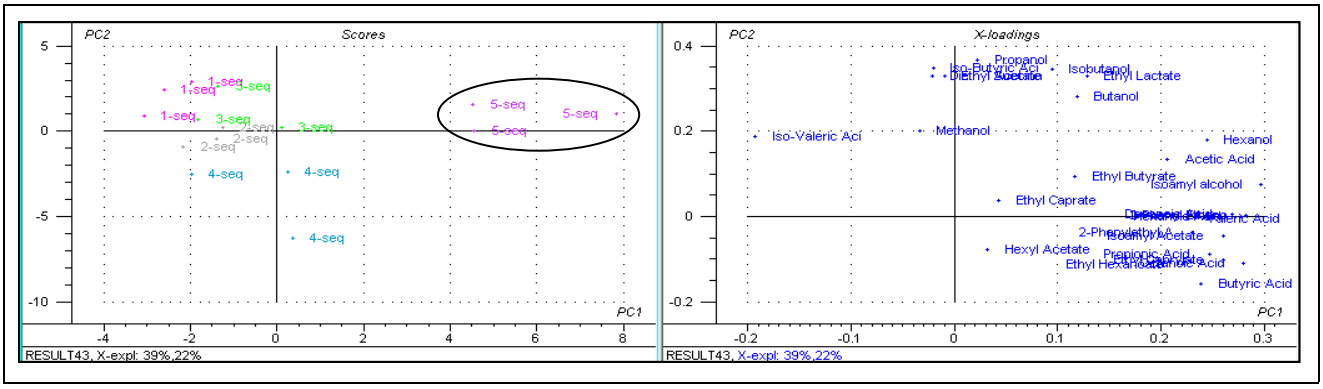

Figure 4.27 PCA score plot (PC1 vs. PC2) generated with the GC-FID data of the MLF treatment samples in sequential inoculation in Pinotage in 2009. PC1 explains 39% of the variance in the data structure.

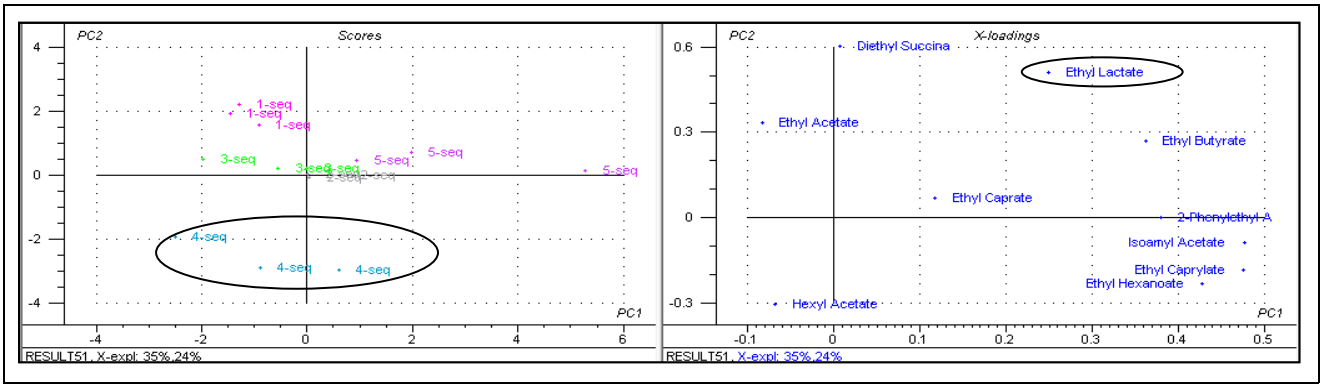

Figure 4.28 PCA score plot (PC1 vs. PC2) generated with the GC-FID data of esters present in the MLF treatment samples in sequential inoculation in Pinotage in 2009. PC1 explains 35% of the variance between the samples.

In PCA's performed on the Cabernet Sauvignon samples, there were very few discernable discriminations observed between the treatment samples. Generating a PCA with the GC-FID data allowed for the discrimination of the ML01 treatment as a result of higher loadings of ethyl acetate, methanol, decanoic acid, 2-phenylethyl acetate and isobutanol. Loadings of the remainder of the volatile compounds seem to allow for the separation of the three mixed culture treatments along

the first PC. A PCA with discrimination based on the higher alcohol content measured with GC-FID, showed that the LAB treatments separated from the yeast ML01 treatment along PC1. Higher loadings of isobutanol was correlated with ML01, while the remainder of the alcohols were responsible for separation of the remaining treatments along PC1 (**Figure 4.29**).

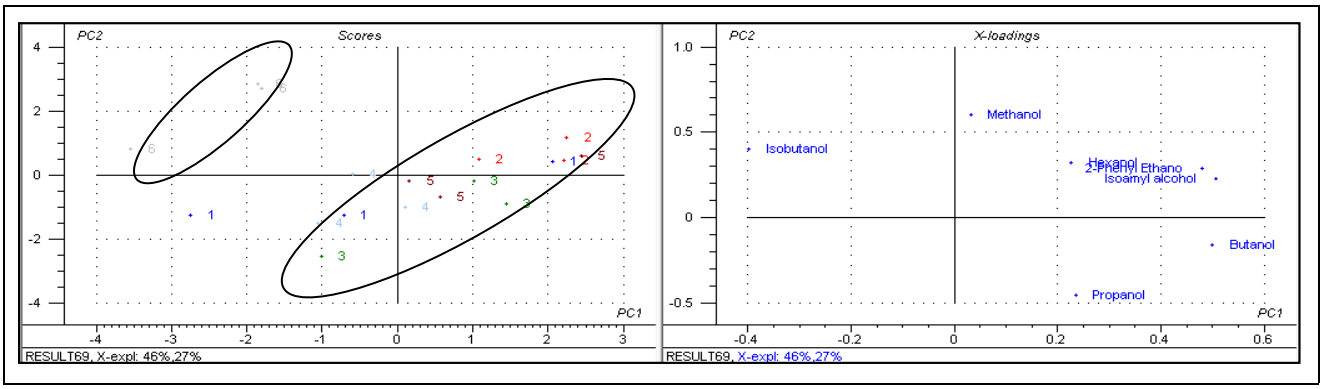

Figure 4.29 PCA score plot (PC1 vs. PC2) generated with the GC-FID data of higher alcohols present in the MLF treatment samples in co-inoculation in Cabernet Sauvignon in 2009. PC1 explains 46% of the variance between the samples.

There were clear groupings and separation of the treatments observed in the Chardonnay and these results were observed in PCA's generated with the volatile compounds. Differences associated with the ester content in the samples, can be seen in the score plot in **Figure 4.30**. Separation of treatment 5 (ML01) along the first PC is correlated with diethyl succinate and ethyl caprate, while the higher loadings for ethyl lactate is negatively correlated with treatment 4, containing *O. oeni* E53 as part of the mixed culture. The other two mixed cultures, treatments 1 and 2 seem to have a similar ester profile compared to the commercial culture VP41 (treatment 4). On the score plots in **Figures 4.31** and **4.32**, the ML01 samples separate from the other treatments along the first PC. Separation of the remaining treatments along the second PC is as a result of higher loadings for methanol, butanol, isoamyl alcohol, hexanol, propanol, isobutanol, iso-valeric acid, acetic acid, propionic acid and iso-butyric acid.

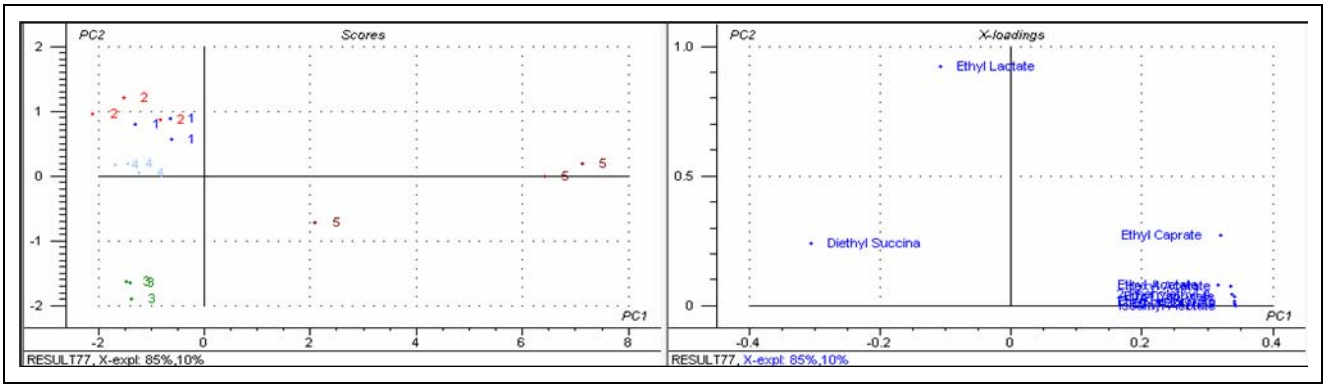

Figure 4.30 PCA score plot (PC1 vs. PC2) generated with the GC-FID data of esters present in the MLF treatment samples in co-inoculation in Chardonnay in 2009. PC1 explains 85% of the variance between the samples.

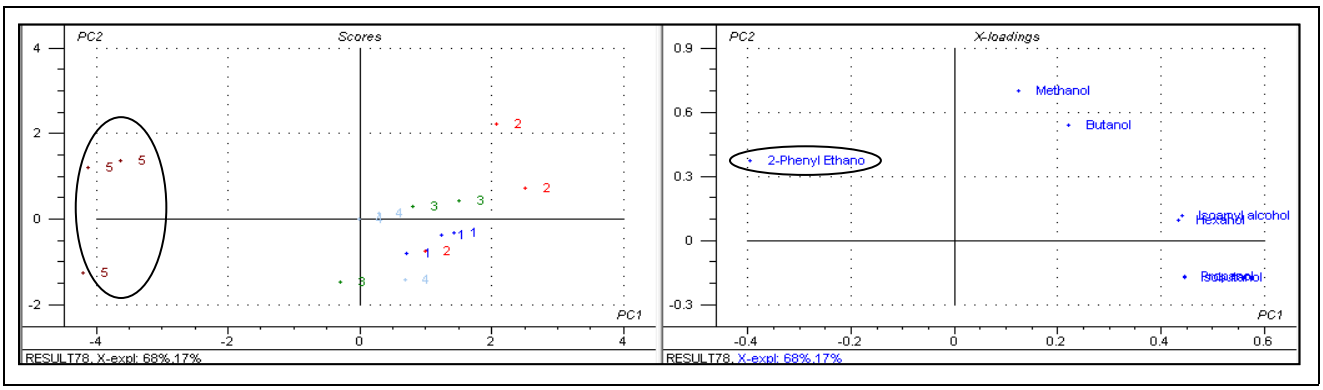

Figure 4.31 PCA score plot (PC1 vs. PC2) generated with the GC-FID data of higher alcohols present in the MLF treatment samples in co-inoculation in Chardonnay in 2009. PC1 explains 68% of the variance in the data structure.

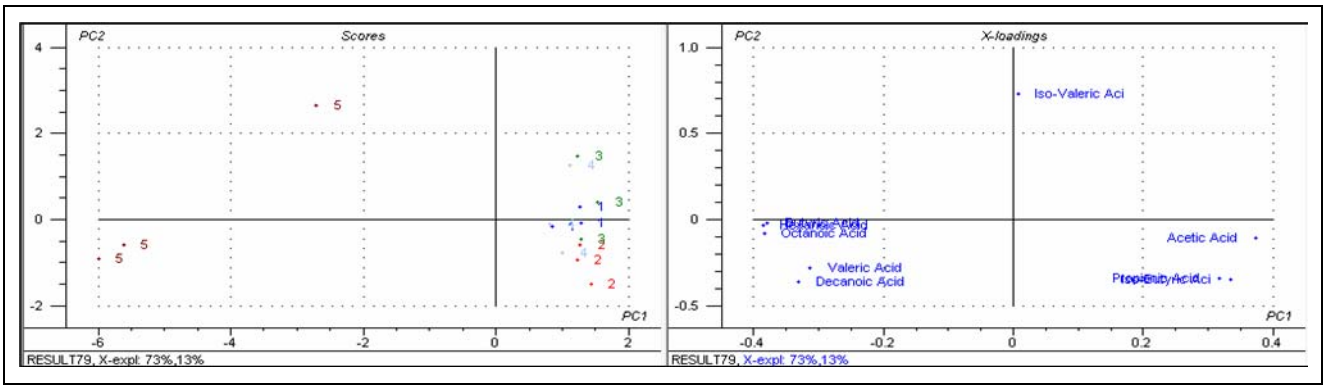

Figure 4.32 PCA score plot (PC1 vs. PC2) generated with the GC-FID data of volatile fatty acids present in the MLF treatment samples in co-inoculation in Chardonnay in 2009. PC1 explains 73% of the variance between the samples.

From the results obtained in this study, there are clear differences in the ability of LAB to alter the aroma profile of the wine and also to what extent this modification will take place. The mixed bacteria cultures differ in their production of volatile aroma compounds. The timing of inoculation also influences the ability of LAB to produce and modify aroma compounds.

4.3.4 INFORMAL SENSORIAL EVALUATION

The first aim of the sensorial evaluation in 2008 was to determine if there were differences between the wines that had been produced with the different combinations of mixed LAB cultures and to generate descriptors associated with these wines. The second and primary aim was the selection of combinations that were to be evaluated in the following vintage and under different inoculation scenarios and this was based on panel-indicated preference.

Panel members agreed that there were definite differences between the different mixed cultures and all the wines that had been fermented with the mixed cultures had better mouthfeel properties than the wine produced with the commercial starter culture VP41. All of the treatment wines were preferred to the spontaneous fermentation control that had not undergone MLF. This is

in agreement with findings by Jeromel *et al*. (2008) and Herjavec *et al*. (2001). These authors observed that MLF wines were preferred compared to non-MLF wines. The wines were also found to be more round and full in taste while wines in which MLF was suppressed, were inferior compared to wines that were subjected to MLF.

Based on deliberation by key panel members, the following combinations were selected for evaluation in the 2009 vintage and for possible use as a commercial malolactic starter culture: *O. oeni* S5 with *Lb. plantarum* 56, *O. oeni* S6 with *Lb. plantarum* 107 and *O. oeni* E53 with *Lb. plantarum* 14.1. These cultures were selected based on their aroma, flavour and taste properties. General descriptors generated by the panel for these three combinations include red berries, fruity, better mouthfeel, well balanced and pleasant aroma.

4.4 CONCLUSIONS

Malolactic starter cultures are used in inoculating for MLF in most red wine cultivars and a few select white wine cultivars. The available starter cultures all contain *O. oeni* as the single LAB culture. This section of the study focused on characterising individual LAB strains in the wine environment for possible use as a starter culture, as well as evaluating mixtures of these isolates for their ability to conduct MLF in various cultivars.

The mixed cultures were able to retain sufficient cell numbers in the wine environment and successfully complete MLF. The mixed cultures differed in their fermentation rates, volatile acidity production and the production of volatile aroma compounds. One of the main modifications associated with LAB metabolism is the affect on the wine aroma. The characterised strains were evaluated for their ability to alter the aroma profile and informal sensorial evaluations were conducted to determine to what extent these modifications contributed to the sensory profile of the wine. Results in this study show that the mixed cultures resulted in diverse volatile aroma profiles, with contributions from both the *O. oeni* and *Lb. plantarum* strain. The differences in aroma compound production associated with the different bacterial cultures could be a valuable tool in producing certain types and styles of wine.

Mixed cultures that showed potential as possible starter cultures were selected for further evaluation, including under different inoculation scenarios. The inoculation time also had an influence on the contribution of the MLF cultures to wine aroma. Mixed cultures also demonstrated compatibility with the two yeast strains used in this study under both co-inoculation and sequential inoculation scenarios. It is necessary for future studies to include more red wine cultivars commonly used in South Africa and to evaluate the compatibility of the bacterial cultures with other yeast strains. Further research is also needed to evaluate the different inoculation scenarios and to generate more data on the effect that the inoculation time has on bacterial performance.

The three mixed cultures selected at the completion of this study all possess the potential to be used as commercial starter cultures for MLF.

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Chapter 5

General discussion and conclusions

5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 CONCLUDING REMARKS AND FUTURE WORK

Malolactic fermentation (MLF) is a secondary fermentation whereby lactic acid bacteria (LAB) convert L-malic acid to L-lactic acid and carbon dioxide. This process is beneficial in wine due to the decrease in acidity and increase in pH, the resulting microbiological stability due to the removal of malic acid as a potential carbon source for microorganisms as well as the changes in the aroma and mouthfeel properties associated with MLF. LAB species from the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* are generally associated with this practice, specifically *Oenococcus oeni*. *Oenococcus oeni* has demonstrated the ability to survive in the harsh wine environment and is the LAB species used in all commercially available starter cultures (Wibowo *et al*., 1985; Davis *et al*., 1988; Kunkee, 1991; Lonvaud-Funel, 1999; Maicas *et al*., 1999; Liu, 2002; Ugliano *et al*., 2003). Malolactic starter cultures are used to initiate MLF in most red wine cultivars and a few selected white wine cultivars. Winemakers are starting to realise the importance of and advantages associated with inoculated MLF compared to the risks related to spontaneous or uncontrolled MLF. Recently, research focus has also started to include the investigation of *Lactobacillus plantarum* and its potential application in wine production (Drici-Cachon *et al*., 1996; Lonvaud-Funel, 1999; G-Alegría *et al*., 2004; Pozo-Bayón *et al*., 2005).

The overall objective of this study was to address the lack of commercial starter cultures containing LAB isolates from South African wine. Many of the commercially available starter cultures are not optimal for use under the harsh South African wine conditions. This is also the first study investigating the possible use of a mixture of LAB cultures for use as a MLF starter culture. The main aim of this study was to develop a starter culture using a mixture of LAB strains from our research environment that are indigenous to South African grapes and wines. This was done by characterising bacterial strains for traits and characteristics of importance in wine quality. Some of these traits included the inability to produce biogenic amines, as well as the ability to survive in the wine environment and retain sufficient microbial populations to degrade malic acid and make a positive contribution to the wine aroma profile (Bou and Powell, 2006).

The first aim of this study was to characterise LAB isolates from the Institute for Wine Biotechnology culture collection as well as strains isolated from spontaneous MLF in Pinotage. These isolates were identified as either *O. oeni* or *Lb. plantarum* and evaluated in a synthetic wine medium for their ability to degrade malic acid. The strains that were successful in degrading malic acid in the synthetic wine medium were screened for genes pertaining to biogenic amine production and were found to contain none of the genes associated with histamine, tyramine or putrescine production. This is an important screening requisite due to the potential health and aroma impact of these compounds (Shalaby, 1996). The following step was to investigate the potential contribution of the LAB isolates to the wine aroma profile by genetic screening of the

enzymes associated with aroma modification during MLF. Results in this study confirmed results found by Mtshali (2007) and Mtshali *et al*. (2009) that the *Lb. plantarum* strains had a more complex enzymatic profile and *O. oeni* strains screened in this study did not possess the gene encoding for *β*-glucosidase. These findings are significant in investigating the possible aroma contribution by the different LAB genera as well as the ability to liberate potential volatile aroma compounds from grape constituents (D'Incecco *et al*., 2004). Future studies should focus on characterising the enzymatic activity, specifically under wine conditions, and correlating this to the analytical data regarding the aroma compounds of the wine. These results, in correlation with the sensory threshold values of the major aroma compounds, will provide a more accurate representation of the aroma contribution of LAB during MLF.

The subsequent stage of the study focused on evaluating the successfully screened strains under winemaking conditions, including different vintages, inoculation times and cultivars. The individual *O. oeni* and *Lb. plantarum* isolates were evaluated in Pinotage in the 2008 vintage. Based on their ability to survive in the wine, as well as their fermentation capabilities, three strains of each of *O. oeni* and *Lb. plantarum* were selected for evaluation as mixed cultures for MLF.

During this study we found that a starter culture containing a mixture of LAB cultures were able to successfully complete MLF in Pinotage, Cabernet Sauvignon, Shiraz and Chardonnay, but further research is needed focusing on other red wine cultivars that are also subjected to MLF. These bacterial cultures were able to successfully degrade malic acid in two inoculation scenarios, sequential- as well as co-inoculation. The latter resulted in the fastest completion of MLF. MLF, in both inoculation scenarios, did not result in an excessive increase in volatile acidity. The mixed cultures were able to maintain sufficient microbial populations until the completion of MLF and displayed fermentation rates comparable to that of the commercial starter culture used as a control in this study. The LAB isolates were compatible with the commercial yeast strains used in this study. No immediate or drastic decrease in cell counts was observed after inoculation for MLF, which imply that metabolites and by-products produced by the wine yeast did not have a detrimental effect on the bacteria. Further investigation is required to elucidate the compatibility of the bacterial isolates with other wine yeast strains as well as the influence of different inoculation regimes on the yeast-bacteria interaction.

The next section of the project focused on the analytical and sensorial evaluation of the wines produced with the mixed cultures. These results, in conjunction with the fermentation data, were utilised in selecting three mixed cultures that were further evaluated in the 2009 vintage. The final aim of the project was to use multivariate data analysis techniques to investigate underlying trends in the datasets concerning the aroma compound production during MLF. In the present study, the concentrations of most volatile compounds were higher in one cultivar than in the others, or even absent in one of the cultivars and produced in others. However, due to the fact that the production of these volatile compounds are dependant on the LAB treatment, cultivar, vintage and timing of inoculation, the compounds that exhibit noticeable trends between different MLF treatments

requires further and more focused research efforts. Generally it was found that MLF, as well as the particular bacterial culture selected for MLF, had a direct effect on the eventual sensory profile of the wine. The amount of total esters produced seems to be influenced by the particular *O. oeni* strain present in the mixed culture. The volatile fatty acids produced were influenced by the *Lb. plantarum* strain present, and in some cases the *O. oeni* strain, while the higher alcohol concentrations produced during MLF were dependant on the strain of *Lb. plantarum* present in the mixed culture. During this study, MLF generally resulted in an increase in ethyl lactate and diethyl succinate, which correlates to the mouthfeel and fruity aromas, respectively, associated with the wine aroma profile after MLF (Francis and Newton, 2005). It is clear from the results obtained in this study that different bacterial strains and mixed cultures can result in different aroma profiles in the final wine. This study generated preliminary results regarding the effect of inoculation timing on the volatile aroma profile in Pinotage in 2009 and should be further investigated in other cultivars and over a number of vintages. Results obtained in this study confirm the profound effect that MLF has on the wine aroma profile, as well as the effect that a specific LAB strain can have on the volatile aroma compound production. Information on the characterised aroma profiles associated with the different bacteria cultures, inoculation times and cultivars evaluated in this study, make it possible to produce a certain style of wine by selecting a specific bacteria culture for MLF. 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. Correlating descriptive sensory analysis with consumer-generated sensory data will provide valuable information regarding the sensory and flavour characteristics that play a role in preference. This can provide a pivotal tool for the winemaker to produce wines fit for a consumer-driven market.

This study generates a number of future research prospects. These include the evaluation of the mixed bacteria cultures in other commonly used South African red grape varieties as well as focusing on the effect of different inoculation times on bacterial performance. The aroma changes associated with the individual isolates in the mixed culture need to be investigated and can provide crucial information concerning the specific contributions of the individual *O. oeni* and *Lb. plantarum* strains. Results from this study also raises the possibility of a starter culture containing a mixture of *O. oeni* strains or a mixture of *Lb. plantarum* strains, based on the same principal as a mixed yeast culture.

Interesting areas of research still to be explored include the population dynamics between the mixed LAB cultures using new and improved technologies and techniques such as randomly amplified polymorphic DNA (RAPD) analysis and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) (Bartowsky *et al*., 2003; Renouf *et al*., 2006; Spano *et al*., 2007).

Findings in this study clearly show that a mixed culture comprising of *O. oeni* and *Lb. plantarum* is able to successfully complete MLF as well as being able to make a positive

contribution to the final wine aroma profile. This study generated three possible mixed MLF starter cultures which can now be evaluated in a commercial winemaking environment.

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