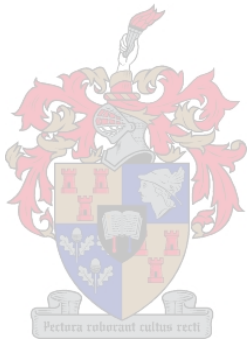
# The evaluation of malolactic fermentation starter cultures under South African winemaking conditions

by

**Hanneli van der Merwe**

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# DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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# SUMMARY

With ever increasing pressure on wine producers to lower the financial costs involved in winemaking to be able to compete in the market, all while maintaining a high level of wine quality, the focus on maintaining control over all aspects of the winemaking process are greatly emphasized.

Malolactic fermentation (MLF) is one of the important processes in red wine production. The advantages of this process, when performed successfully, is widely known and accepted. One way to gain control over MLF is the use of MLF starter cultures. Starter cultures usually consist of *Oenococcus oeni* that has been isolated from grapes or wines and is in most cases available in a freeze-dried form ready for direct inoculation into the wine when MLF is desired. Starter cultures are induced into wine and usually ensure the immediate onset as well as a fast and clean execution of the process. Starter cultures used in South Africa are in most cases isolated from cooler viticultural regions in the Northern hemisphere. The constitution of wines from cooler viticultural regions, differ from those in South Africa, which has a warm climate. The most important difference is the acid content of the wines which is lower in South African must/wines and results into a higher pH. The three most important changes that develop in wine during MLF are a decrease in acidity due to the conversion of malic acid to the less harsh lactic acid, enhanced flavour and aroma of wine and an increase in the microbiological stability of wine. The decrease in acidity is very important for wines produced for grapes grown in cool viticulture regions. In South Africa though, the climate is warm and higher pH's are present in the musts and wines and the de-acidification due to MLF is not the main aim but rather the microbiological stabilisation. One of the compounds that could be produced by lactic acid bacteria (LAB) is biogenic amines (BA's). These compounds can be hazardous to human health. This thesis focussed on the performance of MLF starter cultures in high pH South African red wines.

The first objective of the study was to stretch MLF starter cultures in high pH red wines of South Africa. Stretching means to use less than the prescribed dosage or the re-use of starter cultures. The difference in MLF rate, the influence of the natural occurring LAB and the levels of biogenic amines formed during MLF were determined for the different stretching treatments. The results showed that different rates in malic acid degradation were experienced between the treatments, but in all cases MLF fermentation was

completed. Biogenic amines were formed at various levels and the influence of the natural occurring LAB also played a role.

The second objective of the study was the evaluation of the effect of a wine isolated LAB (*Lactobacillus*) and an acetic acid bacteria (AAB), inoculated with a MLF starter culture had on MLF at different wine pH's. It was found that especially in the case where the *Lactobacillus* was inoculated in combination with the MLF starter culture a possible stimulatory effect was experienced with regards to malic acid degradation rate. Biogenic amine concentration was measured at the end of MLF and it was found that no histamine and tyramine were formed in any of the treatments, while the putrescine and cadaverine levels were found to be at approximately similar levels for the different treatments.

The third objective was to evaluate the possible influence of commercial tannin additions and a pectolytic enzyme on rate of MLF and phenolic composition of high pH red wine. The commercial tannins had possible inhibitory as well as stimulatory effects on the rate of malic acid degradation especially during the initial stages of MLF, with the highest dosage having the significant effect. The BA results showed difference in the levels produced due to tannin additions as well as strain differences could exist. The phenolic content showed a decrease in colour density, total red pigments, total phenolics and anthocyanins between AF and MLF.

The fourth objective was to evaluate inoculation time of MLF starter cultures. The results showed that the fastest AF/MLF time was with simultaneous inoculation of the yeast and MLF starter cultures. It was also for this treatment where no histamine or tyramine was detected at the end of MLF compared to the other inoculation strategies (before the end of AF and after AF).

This study generated a large amount of novel data which made a valuable contribution with regards to MLF in high pH red wines of South Africa.

# OPSOMMING

Die druk om wyne van hoë gehalte teen lae insetkoste te lewer om deel te bly van ’n kompeterende mark, plaas die fokus weer sterk op onder andere die beheer van alle aspekte van die wynmaak proses.

Appelmelksuurgisting (AMG) is een van die belangrikste prosesse van rooiwyn produksie. Die voordele van AMG, in die geval van die suksesvolle implementering daarvan is vandag bekend en word geredelik aanvaar. Een van die metodes om beheer te verkry oor the proses van AMG is deur die gebruik van AMG aanvangskulture. AMG aanvangskulture bestaan uit *Oenococcus oeni* wat geïsoleer word vanaf druiwe of mos/wyn en is in meeste gevalle beskikbaar in ’n gevries-droogte vorm wat direk in wyn geïnokuleer kan word. Aanvangskulture word in wyn geïnduseer om die onverpose aanvang van AMG te bewerkstellig asook om ’n vinnige en skoon deurvoering van die proses te verseker. Die aanvangskulture wat in Suid-Afrika vir hierdie doeleinde gebruik word is in meeste van die gevalle verkry uit koue wingerdbou gebiede in die Noordelike Halfrond. Die samestelling van druiwe van koue wingerdbou gebiede en dié van Suid-Afrikaanse warm wingerdbou gebiede verskil. Die belangrikste verskil word ervaar in die suur inhoud, wat laer is in Suid-Afrikaanse druiwe en dus lei tot ‘n hoër pH inhoud. Die drie mees belangrikste veranderinge wat gedurende AMG in wyn plaasvind is die vermindering van die suur, as gevolg van die omskakeling van appelsuur na melksuur, die verbetering van die aroma en geur van wyn en die verbeterde mikrobiologiese stabiliteit. Die afname in suur is veral belangrik in wyne van koue wingerbou gebiede omdat die suur-inhoud daarvan soveel hoër is. In Suid-Afrika kan hierdie verlaging in suur egter lei tot ’n verdere verhoging in die pH wat plat wyne en uiteindelik ’n verlaging in die kwaliteit van wyn tot gevolg kan hê. Biogene amiene (BA) is verbinding wat melksuurbakterieë (MSB) kan vorm gedurende AMG en kan ernstige implikasies hê vir die mens se gesondheid.

Hierdie tesis fokus op die evaluering van AMG aanvangskulture in hoë pH rooi wyne van Suid-Afrika.

Die eerste doelwit gedurende hierdie studie was om AMG kulture te rek en die invloed daarvan in hoë pH rooiwyn te evalueer ten opsigte van the tempo van AMG, die rol van die natuurlike MSB te bestudeer asook om die vlak van biogene amiene te bepaal vir die verskillende behandelings. Die resultate het aan die lig gebring dat die rek van kulture

verskille in die tempo van appelsuur afbraak tot gevolg het, maar dat AMG in alle gevalle wel suksesvol deurgevoer kon word. Die BA'e wat gevorm is, was teenwoordig in verskillende hoeveelhede.

Die tweede doelwit was om die effekt van die gesamentlike inokulasie van 'n wyn geisoleerde MSB (*Lactobacillus*) asook 'n asynsuurbakterie (ASB) met 'n kommersiële AMG aanvangskultuur op AMG te evalueer. Hierdie eksperiment is uitgevoer by verskillende pH's. Daar is gevind dat veral in die kombinasie inokulasie met die *Lactobacillus*, die tempo van appelsuur afbraak moontlik gestimuleer was. Geen histamien of tiramien is tydens AMG gevorm in hierdie eksperiment gevorm nie, terwyl putresien en kadaverien teenwoordig was teen ongeveer gelyke vlakke vir die behandelings.

Die derde doelwit was om die moontlike invloed van kommersiële tannien toevoegings en die toevoeging van 'n pektolitiese ensiem te evalueer ten opsigte van AMG tempo die fenoliese samestelling van rooiwyn te bestudeer. Verskillende kommersiële tanniene het 'n moontlike sowel as inhiberende uitwerking gehad, veral gedurende die aanvanklike stadium AMG. Die grootste verskille is waargeneem in die behandelings waar die hoogste dosisse tannien bygevoeg is. Die BA resultate toon dat verkillende vlakke geproduseer was en dat hierdie verskille onstaan het as gevolg van verskille in tannien dosisse sowel as aanvangskulture. Die fenoliese inhoud het 'n afname in kleur intensiteit, totale rooi pigmente, totale fenole en antosianiene getoon vir die periode vanaf AF tot die einde van AMG.

Die vierde doelwit was om the tyd van inokulasie van AMG aanvangskulture te bestudeer. Die resultate het getoon dat die vinningste tydperk van AF/AMG was ondervind in die geval waar die gis aanvangskulture gelyktydig met die AMG aanvangskulture geïnokuleer was. Geen histamine en tyramine het ook in hierdie behandeling ontwikkel nie, terwyl daar wel vlakke teenwoordig was in die ander behandelings (inokulasie net voor die einde van AF en na afloop van AF).

Tydens hierdie studie is 'n groot hoeveelheid nuwe data geskep wat 'n groot bydrae ten opsigte van AMG in hoë pH rooi wyne vanaf Suid-Afrika kan lewer.

This thesis is dedicated to my husband Renier van der Merwe and my parents, Fanus and Friedel Walters.
Hierdie tesis is opgedra aan my man, Renier van der Merwe sowel as my ouers, Fanus en Friedel Walters.

# BIOGRAPHICAL SKETCH

Hanneli van der Merwe (neé Walters) was born in De Aar, South Africa on 8 May 1981. She matriculated at Ladismith High School in Ladismith in 1999. Hanneli enrolled at Stellenbosch University in 2000 and obtained a BScAgric degree, majoring in Viticulture and Oenology, in 2004. In 2005, she enrolled for an MScAgric degree in Oenology at the same University.

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# PREFACE

This thesis is presented as a compilation of 7 chapters. Each chapter is introduced separately.

**Chapter 1** **General Introduction and Project Aims**

**Chapter 2** **Literature Review**
Malolactic fermentation

**Chapter 3** **Research Results**
The stretching of malolactic fermentation starter cultures in high pH red wines

**Chapter 4** **Research Results**
Effect of a wine isolated *Lactobacillus* spp. and an *Acetobacter pasteurianus* in combination with a malolactic fermentation starter culture on MLF at different wine pH's

**Chapter 5** **Research Results**
The effect of commercial tannins and a pectolytic enzyme on malolactic fermentation and phenolic composition of red wine

**Chapter 6** **Research Results**
Assessing different inoculation times of malolactic fermentation in high pH red wines

**Chapter 7** **General Discussion and Conclusions**

# CONTENTS

# 1. GENERAL INTRODUCTION AND PROJECT AIMS

## 1.1 INTRODUCTION

With a history that dates back to 2 February 1659 (Thom, 1958), the South African wine industry has developed and adapted to become a strong competitor in the international wine arena of quality produced wines. Today the emphasis falls on developing even more efficient production methods to facilitate the overall production process and meeting consumer demands, yet maintaining a high quality of wine production.

The two most important biological processes during winemaking where control can be exerted are alcoholic fermentation (AF) and malolactic fermentation (MLF).

MLF is required in the production of almost all red wines as well as certain white and sparkling wines. MLF is performed by lactic acid bacteria (LAB) containing the malolactic enzyme (MLE). It practically refers to a biological process of wine deacidification in which the dicarboxylic L-malic acid is converted to the monocarboxylic L-lactic acid with the production of $CO_2$ (Davis *et al*., 1985). This deacidification as a result of MLF is very favourable for wines produced in cool viticultural climates such as occurs in Germany, France and the Eastern United states (Beelman and Gallander, 1979; Kunkee, 1967, 1974; Rice, 1974). However, in wines with high pH produced in the warmer viticultural regions for example California, South Africa and Australia it can lead to insipid, flat wines and the growth of spoilage bacteria (Rankine, 1971, 1972). As fermentative organisms, LAB can also catabolise sugar to form lactic acid (major end product) and other flavour compounds for example, acetaldehyde, acetic acid, ethanol, diacetyl, acetoin and 2,3-butanediol, in a variety of fermented products. Diacetyl for example can enhance or reduce the complexity of wines. At concentrations of 1-4 mg/L, it adds to the complexity of wine, whilst between 5-7 mg/L it can cause a buttery aroma which is considered undesirable (Rankine, 1977, Rankine *et al*., 1969). Microbial stability is another possible outcome of MLF, since wines that have undergone MLF are more microbiologically stable than those that have not (Kunkee, 1967, 1974; Rankine, 1972). Extensive research has been done over the years on the process of MLF and its importance in wine quality.

MLF also results in the generation of a high proton motive force, which can drive ATP synthesis. To generate this proton motive force three main modes of transport of L-malate

and L-lactate exits. These are electrogenic malate/lactate antiport, electrogenic malate uptake and electrogenic lactate efflux (Poolman *et al*., 1991).

The LAB that has the ability to conduct MLF is mainly *Oenococcus oeni*, *Lactobacillus* spp. and *Pediococcus* spp. *O. oeni* is the preferred species used to induce MLF commercially due to its acid tolerance (Drici-Cachon *et al*., 1996), increased resistance for high alcohol concentrations (Davis *et al*., 1988; Ribéreau-Gayon *et al*., 1998), higher resistance to $SO_2$ (Henick-Kling, 1988) and flavour profile produced (Kunkee, 1967; Davis *et al*., 1985; Liu, 2002). It have been found by various authors that due to the above-mentioned preferences *O. oeni* is the LAB naturally selected during AF, since it is in most cases the only LAB present after AF.

MLF can occur naturally or be induced using MLF starter cultures. Spontaneous MLF is very unpredictable as it can occur during AF or the onset may be delayed for several months after AF. Starter cultures therefore provide the tool to at least control the onset of MLF and the type of LAB that performs MLF.

Kunkee (1967, 1974) found that there are advantages to controlling the organisms that conduct MLF by pure culture inoculation. This idea sparked various studies into the kinds of bacteria that can perform MLF and the factors that influence MLF.

The first potential starter culture that was studied was ML-34, a *Leuconostoc oenos* (renamed to *O. oeni* by Dicks *et al*. (1995)) strain that was isolated from a Californian red wine by Ingraham *et al*. (1960). PSU-1 was another early strain to be used in studies in connection with pure culture inoculation for MLF and was isolated by Beelman *et al*. in 1977. Since then various starter cultures have been developed and are mostly freeze-dried cultures today. A viability of >95% has been recorded for freeze dried cells (Henick-Kling, 1993). These early freeze dried starter cultures used to require a rehydration or reactivation step before inoculation into wine, but today some can even be directly inoculated into wine.

The optimal time for inoculation with MLF starter cultures has also sparked numerous studies. It depends on various factors which include the type of wine/cultivar, $SO_2$, alcohol content, pH and temperature (Henick-Kling, 1993). Co-inoculation of yeast and MLF starter cultures versus inoculation near the end or after AF were studied by various authors like Grossman *et al*. (2002), Henick-Kling and Park (1994), Jussier *et al*. (2006) and Rauhut *et al*. (2001).

New ways to improve MLF are constantly being researched. Areas that have been investigated include the use of enzymatic reactors (Formisyn *et al*., 1997), genetic engineering of *Saccharomyces cerevisiae* strains to conduct MLF (Ansanay *et al*., 1996; Bauer, 2003; Bony *et al*., 1997; Denayrolles *et al*., 1995; Husnik *et al*., 2006; Volschenk *et al*., 1997), bioreactors based on high biomass of free cells, immobilised cells and on enzymes (Maicas *et al*. 1999, 2001; Maicas, 2001; Diviès *et al*., 1994). The development of new and improved starter cultures is also continuously investigated.

Information regarding the performance of MLF starter cultures, specifically in high pH red wines of SA, and the practical implications thereof for the winemaker and winemaking process still needs some exploration.

## 1.2 SPECIFIC PROJECT AIMS

This study focused on the use of commercial starter cultures in high pH (3.7-4.0) red wines of South Africa. The stretching or re-use of starter cultures, influence of natural LAB and acetic acid bacteria (AAB), the effect of commercial tannins and early inoculation will be evaluated during this study.

Objective 1: The stretching of MLF starter cultures in South African high pH red wines.

Aims:
(1) to evaluate the difference in MLF rate between the different stretching treatments;
(2) to investigate the influence of the naturally occurring LAB within the different stretching treatments; and
(3) determine the levels of biogenic amines formed during MLF for the different stretching treatments.

Objective 2: The evaluation of the effect of lactic acid and acetic acid bacteria in combination with a malolactic fermentation starter culture at different wine pH's.

Aims:
(1) to evaluate the influence of a naturally occurring LAB and an AAB strain on the growth of a MLF starter culture;
(2) to investigate the influence on the MLF rate; and
(3) to determine the levels of biogenic amines formed during MLF for the different treatments.

Objective 3: The influence of commercial tannins and an enzyme on malolactic fermentation in high pH red wines.

Aims: (1) to evaluate the possible effects of commercial tannins and the pectolytic enzyme on MLF rate;

(2) to evaluate the impact on the phenolic composition;

Objective 4: The evaluation of early inoculation of MLF starter cultures

Aims: (1) to evaluate different inoculation times of MLF starter cultures in high pH red wines

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# 2. LITERATURE REVIEW

## 2.1 INTRODUCTION

The winemaking process consists of two fermentations namely, alcoholic fermentation (AF) and malolactic fermentation (MLF). AF is regarded as the primary fermentation and MLF the secondary fermentation. MLF is required during the making of almost all red wines and also during the making of some white ~~and sparkling wines~~, especially those destined to be aged in barrels and sparkling wines. MLF is an important determinant of final wine quality. It can occur spontaneously in wine or can be induced. In either case a ~~, the slightest~~ delay in the onset of this process may lead to an alteration of the wine quality (Henick-Kling, 1995).

This secondary fermentation is based on a decarboxylation reaction where malate is converted to lactate by lactic acid bacteria (LAB) which possess the malolactic enzyme (MLE). In addition to decreasing wine acidity, MLF improves the microbiological stability and the organoleptic characteristics of wines (Davis *et al*. 1988; Kunkee, 1991). These aforementioned organoleptic changes are as a result of secondary bacterial metabolisms (Lonvaud-Funel, 1999). The activity of wine LAB has been studied for more than three decades, with the focus ~~mainly~~ on the malic acid degradation by *Oenococcus oeni* ~~species~~, the pre-dominant species of LAB involved with MLF (Lonvaud-Funel, 1995). Starter cultures for MLF, similar to active dried yeast used to induce AF, have been developed over the past two decades. The starter cultures for MLF that are available today have mostly been isolated from wines~~grapes cultivated~~ in the northern hemisphere which have a different composition than those from South Africa. In South Africa, the main role of MLF is the achievement of microbial stability and improvement of the aroma profiles of wines, whilst in the cooler climate winemaking regions MLF is mainly performed for de-acidification purposes. In South Africa the long hot summers result in musts with higher sugar concentrations and therefore wines with higher ethanol concentrations (14-16% v/v). The higher pH musts (3.4 - 4.0) require higher $SO_2$ additions ~~and cooling~~.

This literature review will focus on the use of starter cultures to perform MLF as well as the factors that influence this process.

## 2.2 LACTIC ACID BACTERIA ASSOCIATED WITH WINEMAKING

### 2.2.1 General information and metabolism of LAB

LABS are generally Gram- positive, aerobic to facultative anaerobic, asporogenous rods and cocci, oxidation-, catalase-, benzidine- and gelatine- negative. TheyLAB also do not have cytochromes, reduce nitrate to nitrite or use lactate.

LAB are functionally related due to the production of lactic acid from glucose. Based on their metabolism of glucose, LAB may be divided into three main groups namely, obligatory homofermentative, facultative heterofermentative and obligatory heterofermentative. Homofermentative LAB reduce hexose sugar to lactic acid via the Embden Meyerhof Parnas (glycolytic pathway) (**Figure 2.1**) whilst heterofermentative lactobacilli, leuconostocs and oenococci produce D-lactic acid and acetic acid through the 6-phosphogluconate pathway (Du Toit and Pretorius, 2000). The main genera of wine LAB are *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus*. The homofermentative cocci are mainly *P. damnosus*, and *P. pentosaceus*. Lactobacilli can be both facultative (*L. plantarum, L. casei*) and obligatory (*L. hilgardii, L. brevis, L. fructivorans*) heterofermentative species. *Leuc. mesenteroides* and *O. oeni* are the heterofermentative cocco-bacilli in wine (Strasser de Saad and Manca de Nadra, 1992; Buckenhüskes, 1993; Caplice and Fitzgerald, 1999; Du Toit and Pretorius, 2000; Mira de Orduña *et al*., 2000).

LAB can also convert malic acid into lactic acid via a unique energy producing pathway. This pathway involves the energy gradient producing transport of malic acid into the cell, intracellular decarboxylation by the malolactic enzyme, and the efflux of lactic acid possibly with one accompanying hydrogen ion. (Henick-Kling *et al.*, 1998; Poolman *et al*., 1991).

Another substrate utilised by LAB is L-arginine, one of the most abundant amino acids in grape must and wine. Heterofermentative LAB can degrade L-arginine to produce ammonia, ornithine, ATP and $CO_2$ via the arginine deiminase (ADI) pathway. The possibility exits that urea could be formed in the arginase-urease pathway (Liu and Pilone, 1998). An intermediate in the ADI pathway, citrulline, is also a precursor for the carcinogenic compound ethyl carbamate (EC). Thus, the use of MLF starter cultures that are non-arginine degrading has been proposed by Mira de Orduña *et al*. (2001). Another important metabolism of LAB is the metabolism of citrate. Citrate is transformed to lactate,

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acetate, diacetyl, acetoin and 2,3-butanediol and a small amount is converted to aspartate via oxaloacetate and aspartate aminotransferase (Liu, 2002).

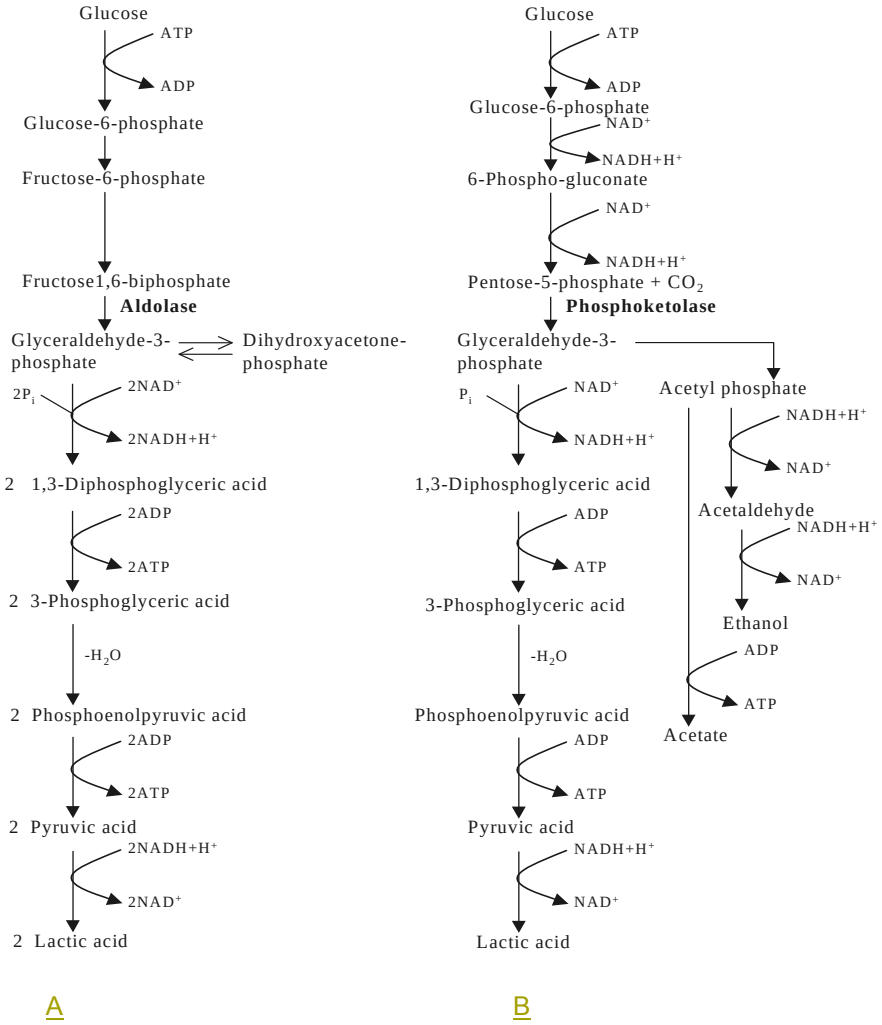


**Figure 2.1** (A) Embden-Meyerhof-Parnas pathway (glycolysis) of homofermentative LAB and (B) 6-phosphogluconate pathway of heterofermentative LAB (adapted from Du Toit and Pretorius, 2000)

Certain LAB are~~is~~ also able to convert certain amino acids in wine into biogenic amines which could have a negative impact on human health when consumed in high amounts (Ten Brink *et al*., 1990).

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### 2.2.2 The evolution of LAB during winemaking

The concentration of viable LAB populations is approximately $10^2$-$10^4$ cells/mL in must from healthy grapes, with variations due to conditions during the final days of ripening and harvest (Lonvaud-Funel, 1995). Grape must, receives $SO_2$ at crushing, which reduces the bacterial populations drastically. At this stage, another factor affecting bacterial populations is the initiation of alcoholic fermentation (AF). It leads to unfavourable conditions for bacterial growth due to an altered chemical and physical environment as well as competition with yeast (Lonvaud-Funel *et al*., 1988).

~~The species of LAB that occur naturally on grapes are *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*.~~ Viable populations of strains of LAB~~these species~~ that are resistant to low pH (<3.5), high $SO_2$ levels (50 ppm) and high ethanol levels are able to survive in wine (Van Vuuren & Dicks, 1993; Lonvaud-Funel, 1999). After alcoholic fermentation the viable LAB cells numbers ~~is~~are approximately $10^2$-$10^3$ cells/mL. *Lactobacillus* spp., *P. damnosus*, *Leuc. mesenteroides* and *O. oeni* predominate during AF but after AF, *O. oeni* (formerly known as *Leuconostoc oenos*) (Dicks et al, 1995) dominates (Lonvaud-Funel *et al*., 1991; Van Vuuren and Dicks, 1993; Lonvaud-Funel, 1999). *O. oeni* is the species that is positively associated with MLF due to its tolerance of low pH (<3.5) and the resultant flavour profile. *Pediococcus* and *Lactobacillus* species will more likely occur in wine with a high pH (3.5-4.0) after MLF and are usually associated with spoilage (Davis *et al*., 1985; Lonvaud-Funel, 1995).

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**Table 2.1: The evolution of LAB species during alcoholic fermentation. Cell numbers expressed as cfu/mL (Lonvaud-Funel, 1995).**

| Species | Day | | | | |
|---|---|---|---|---|---|
| | 0 | 3 | 6 | 10 | 18 |
| *O. oeni* | nd | nd | nd | $4.3X10^3$ | $3.4X10^6$ |
| *Leuc. mesenteroides* | $2.9X10^2$ | $1.7X10^4$ | $9.6X10^4$ | $3.2X10^3$ | nd |
| *P. damnosus* | $6.0X10^2$ | $3.8X10^4$ | $3.7X10^4$ | $4.9X10^3$ | nd |
| *L. hilgardii* | $1.1X10^3$ | $8.0X10^4$ | $4.0X10^4$ | $4.4X10^3$ | nd |
| *L. brevis* | nd | $2.0X10^4$ | $4.5X10^3$ | nd | nd |
| *L. plantarum* | $7.5X10^1$ | $2.0X10^4$ | nd | nd | nd |
| *L. casei* | $7.7X10^1$ | $2.0X10^4$ | nd | nd | nd |
| Total | $2.5X10^3$ | $1.7X10^5$ | $1.5X10^5$ | $1.8X10^4$ | $3.4X10^6$ |

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## 2.3 MALOLACTIC FERMENTATION

### 2.3.1 Process of MLF

MLF refers to the decarboxylation reaction where one molecule of L-malic acid (malate) is converted to one molecule each of L-lactic acid (lactate) and carbon dioxide (Davis *et al.* 1985; Lonvaud-Funel, 1995) (**Fig 2.2**). This conversion is performed by lactic acid bacteria (LAB) that contain the malolactic enzyme (MLE). MLE is the only enzyme involved in MLF and has been purified from various LAB (Lonvaud-Funel and Strasser de Saad, 1982; Caspritz and Radler, 1983; Spettoli *et al*., 1984; Naouri *et al*., 1990). In the presence of $NAD^+$ and $Mn^{2+}$, MLE reacts similar to the malic enzyme combined with lactate dehydrogenase, but without the release of intermediate products. The complete nucleic acid sequence of the *mle* gene has been determine for *Lactococcus lactis* (Denayrolles et al., 1994), *O. oeni* (Labarre *et al*., 1996) and *P. damnosus* (Bauer, 2003).

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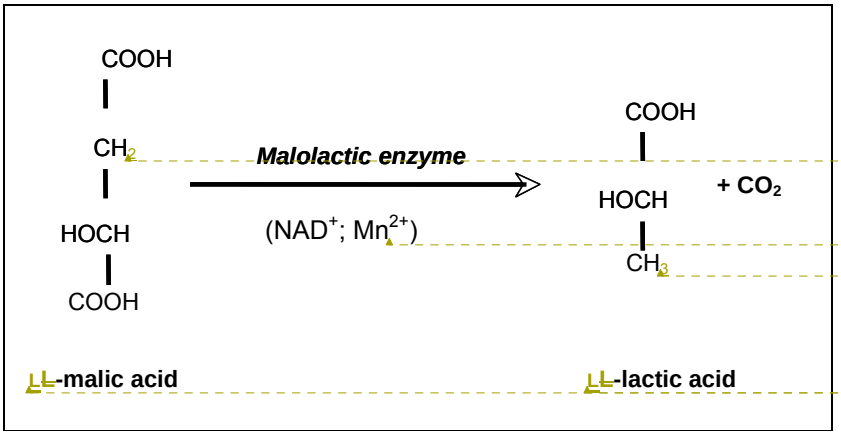


**Figure 2.2** Conversion of L-malic acid to L-lactic acid and $CO_2$ by LAB that contains the MLE, with $NAD^+$ and $Mn^{2+}$ acting as co-factors.

MLF leads to the de-acidification of wine as L-malic acid has a much harsher taste than the L-lactic acid that is produced. This de-acidification is desirable in wines with high acidity from the cooler climate wine producing regions. ~~The conduction of~~ MLF is promoted in wines that are to be aged in barrels, will undergo extended bottle maturation or when certain organoleptic qualities are desired (Bauer and Dicks, 2004). As per example, wines from Germany, France and the Eastern United States which are cool viticultural regions will benefit from the de-acidification due to MLF. Wines from warmer regions such as~~like~~ South Africa, California and Australia have a lower acidity (Davis *et al*., 1985; Kunkee, 1967; Wibowo *et al*., 1985). MLF could be detrimental to these wines, possibly resulting in spoilage by lactic acid bacterial species like p~~P~~ediococci and l~~L~~actobacilli that could subsequently lead to flat, insipid wines (Rankine, 1972; Rankine and Bridson, 1971).

In addition to deacidification, MLF can also lead to ~~definite~~ changes in the organoleptic profile of a wine. The metabolism of various other substrates that were not utilised during alcoholic fermentation may mediate these changes. These products of MLF include mostly lactic acid, acetic acid (volatile acidity), diacetyl (buttery flavour), acetoin, 2,3-butanediol, 2-acetolactate, 2-acetohydroxybutyrate, ethyl acetate and ethyl lactate (as cited by Delaquis *et al*., 2000)(**Figure 2.3~~2~~**).

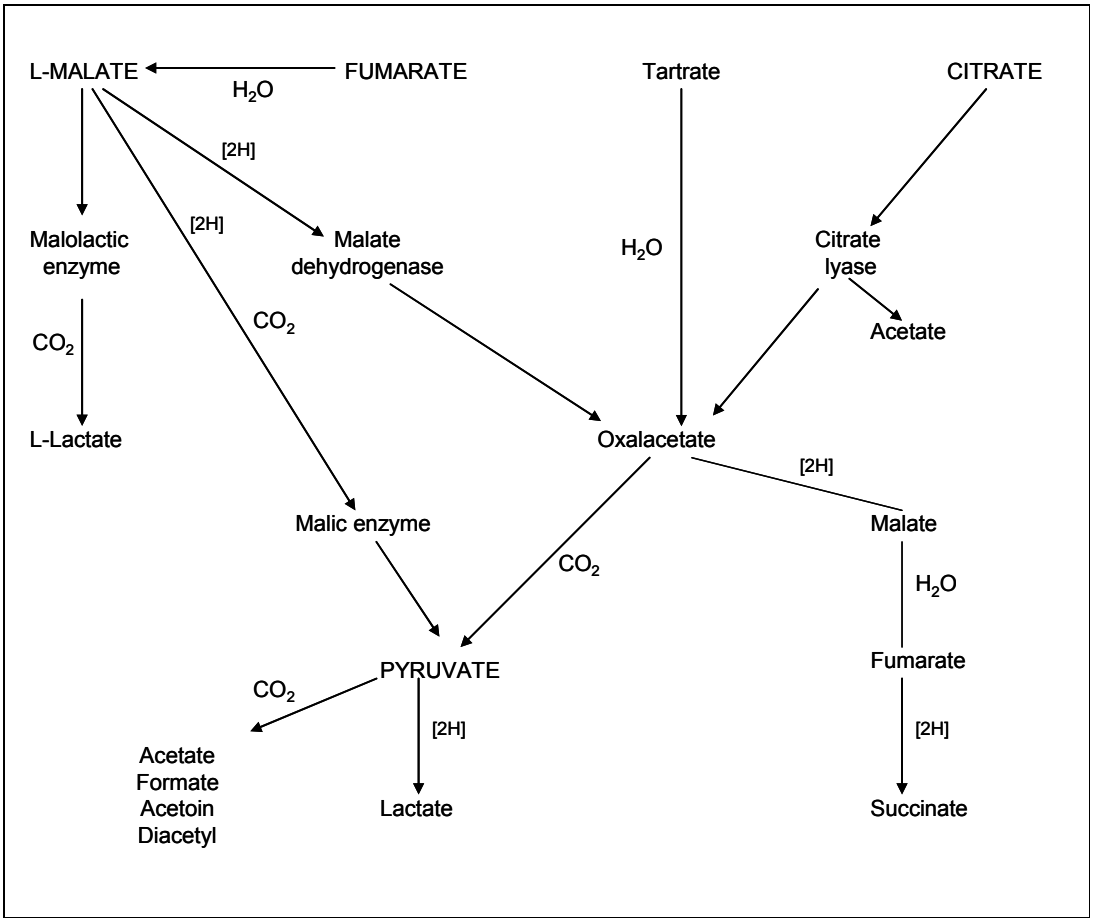


**Figure 2.3.2** Metabolic pathways of LAB leading to important organoleptic compounds (Van Vuuren and Dicks, 1993).

Another effect of MLF is the increased microbiological stability of wine. The explanation for this effect is the depletion of residual nutrients by the LAB during MLF and the production of antibacterial compounds (Lonvaud-Funel and Joyeux, 1993; Rammelsberg and Radler, 1990).

### 2.3.2 Starter cultures

Kunkee (1967; 1974) found that there are advantages to controlling the organisms that conduct MLF by pure culture inoculation, instead of waiting for spontaneous MLF to take its course. This idea sparked various studies into the bacteria that can perform MLF and the factors that influence MLF.

——The first potential starter culture that was evaluated in this regard was ML-34, a *Leuconostoc oenos* strain that was isolated from a Californian red wine (Ingraham *et al*., 1960). ML-34 was first classified as *Leuconostoc citrovorum* (Pilone *et al*., 1966) but after Garvie (1967) re-organised the leuconostocs, Pilone and Kunkee (1972) re-classified it as *Leuconostoc oenos*. PSU-1, isolated by Beelman *et al*. in 1977, was another strain used in early studies concerning pure culture inoculation for MLF.

Since then, various starter cultures have been developed and are either lyophilised or freeze dried cultures, the most effective ones consist of *O. oeni* strains that were originally isolated from wine. **Table 2.2** show some of the malolactic fermentation starter cultures that are available today. The number of MLF starter cultures available on the SA market are much less than yeast starter cultures.

Viability of >95% has been recorded for freeze dried cells (Henick-Kling, 1993). Transport and long term storage of freeze dried cultures could be problematic in wineries if the proper cooling facilities for storage do not exist. A solution to this problem could be to develop MLF starter cultures similarly to the method used to produce commercial yeast starter cultures, which is fluidised-bed drying. Clementi and Rossi (1984) studied the effect of fluidised-bed drying and storage on the survival of *O. oeni*~~*os*~~. The cell viability was largely unaffected by the drying conditions and the total count recorded immediately after drying was a one to two decimal reduction. When stored, the dry cultures had a higher survival rate when kept refrigerated than at room temperature conditions.

**Table 2.2: Malolactic fermentation starter cultures that is commercially available today**.

| Culture | Company | Strain | Characteristic |
|---|---|---|---|
| Viniflora®oenos | Chr.Hansen* | *O. oeni* | High fermentation speed |
| Viniflora®CH16 | Chr.Hansen | *O. oeni* | Tolerance in high alcohol conditions |
| Viniflora®CH35 | Chr.Hansen | *O. oeni* | Tolerance for low pH and high $SO_2$ |
| LALVIN 31 | Lallemand** | *O. oeni* | Tolerance for low pH |
| ENOFERM ALPHA | Lallemand | *O. oeni* | Tolerance for high $SO_2$ |
| LALVIN VP41 | Lallemand | *O. oeni* | Tolerance in high alcohol conditions |
| Biostart Oenos | Erbsloh*** | *O. oeni* | |
| Biostart Bianco SK3 | Erbsloh | *O. oeni* | Low diacetyl producer |
| Microenos B16 standard | Laffort**** | *O. oeni* | White wines |
| Microenos MBR.B1 | Laffort | *O. oeni* | |
| Lactoenos SB3 | Laffort | *O. oeni* | High fermentation rate |
| Lactoenos 450 PreAc | Laffort | *O.oeni* | Extremely tolerant to most wine conditions |

*www.chr-hansen.com
**www.lallemandwine.co.za
***www.erbsloeh.com
****www.laffort.com

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### 2.3.2.1 Preparation for inoculation

~~Lactic acid bacteria starter biomass is mostly produced by manufacturers in rich, synthetic media and often, the bacteria will find it difficult to survive after inoculation into wine.~~ The wine matrix may lack in the complex of nutrients needed by LAB to survive and the chemical (pH < 3.4; Ethanol > 14% v/v) as well as the physical (Temperature < 18ºC) parameters can sometimes be a harsh environment for LAB. Rodriques *et al*. (1990) showed that the cell numbers could decrease~~d~~ by up to three log-cycles when inoculated into wine.

To compensate for this phenomenon, cells should go through a reactivation or re-hydration step before inoculation into wine (as recommended by most manufacturers). Importantly, such a step should be quick and simple, it must not modify the characteristics of the wine in any way and it must bring the cells to a physiological state that will permit their survival and growth in the wine.

Some of the first researches on reactivation and re-hydration showed that in order to prevent the death of cells, reactivation should occur in a medium that is supplemented with yeast extract and grape juice (Lafon-Lafourcade *et al*., 1970; ~~as cited by Bauer and Dicks, 2004);~~ Lafon-Lafourcade *et al*., 1983). Fornachon (1968) and Mascarenhas (1984) showed that nutrients produced during yeast autolysis may stimulate the growth of MLF bacteria, whilst Gallander (1979) obtained poor growth in the presence of yeast extract. Media enriched with 40-80% wine could also be used to activate/enhance the growth of *O. oeni* (Davis *et al*., 1985) or yeast (Kunkee, 1967).

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Nault *et al*. (1995) studied the duration of the reactivation process as well as the initial reactivated cell population. They found that reactivation in half strength wine followed by growth in a medium with 75% wine allowed a gradual readjustment of bacteria to counter balance the effect of wine component. They also showed that the pre-culture mediuma has to be, at most, $10^7$cfu/mL so that malic acid degradation follows cellular proliferation.

In a study by Semon *et al*. (2001) it was found that the rate of MLF did not vary according to the method by which bacterial starter cultures were prepared (re-hydration of freeze-dried forms or prior growth in diluted grape juice).

Today the inoculation of MLF bacterial starter cultures is very easy and fast. Some of the MLF bactaria manufacturers have products that can be added directly to wine without re-hydration, for instance a typical inoculation protocol entails adding the cultures in its granulated form directly into wine, or it may be dissolved in a smaller volume and then added to the larger volume (Chr. Hansen~~, Lake Internation technologies~~; Lallemandd, ~~South Africa~~). Typical preservation of the cultures are to store it at +5 ºC to preserve it for 6 months, or to preserve it for 36 months the storage temperature should be -18 ºC. ~~Starter cultures like these have been developed through subjecting the cultures to various inhibitory conditions like could occur in wine, during the production process.~~

### 2.3.2.2 Inoculation time

The optimal time for inoculation is influenced by various factors which include the cultivar, wine type, $SO_2$ and alcohol content, pH and temperature (Henick-Kling, 1993). Inoculation at the end of AF is common practice amongst wine makers globally, but this may lead to a delay in the onset of MLF due to high ethanol concentrations (Lafon-Lafourcade *et al*., 1983; Davis *et al*., 1985). ~~The time of inoculation is another important factor for a successful MLF that has been studied over the last two decades. In 1979 Gallender~~

showed that inoculation during or after alcoholic fermentation was most favourable for the stimulation of malolactic fermentation. Inoculation at the end of AF is common practice amongst wine makers globally, but this may lead to a delay in the onset of MLF due to high ethanol concentrations (Lafon-Lafourcade *et al*., 1983; Davis *et al*., 1985). There have been various arguments in favour of inoculation after AF, because it could prevent the possible antagonism with yeast and production of undesirable metabolites (Lafon-Lafourcade et al., 1983; Ribéreau-Gayon, 1985, Henick-Kling and Edinger, 1994). Inoculation of the bacteria during alcoholic fermentation is preferred by some winemakers (Davis *et al*., 1985; Gallander, 1979). The rationale behind this is that at this stage most of the free $SO_2$ is bound by organic acids produced during yeast growth (Davis *et al*., 1985). The optimal time for inoculation is influenced by various factors which include the variety of wine/cultivar, $SO_2$ and alcohol content, pH and temperature (Henick-Kling, 1993).

## 2.4 FACTORS THAT INFLUENCE MALOLACTIC FERMENTATION

There are various chemical and physical factors that influence the successful completion of MLF in wine. The four most important factors are pH, temperature, alcohol content and $SO_2$ and other factors are carbohydrates, L-malate, L-lactate, citrate, other organic acids (tartaric acid, succinate), fatty acids, amino acids oxygen and carbon dioxide, acetaldehyde, phenolic compounds, pesticides, availability of nutrients and pre-culture conditions.

### 2.4.1 The influence of pH

Davis *et al*. (1986) showed that the growth rate of *O. oeni* increased as the wine pH increased from 3.2 - 4.0, and MLF occurred in conjunction with growth, this was confirmed in a study by Wibowo *et al*. (1988), who showed that the rate of MLF, conducted by *O. oeni*, increased as wine pH increased from 3.1-3.8. It has also been shown by Davis *et al*. (1986, 1988), that *O. oeni* is the species of LAB with the greatest tolerance to low pH values, since this species is almost exclusively isolated at a pH < 3.5. High pH (>3.5) contains more species of *Lactobacillus* and *Pediococcus* (Davis *et al*., 1986, Du Toit and Pretorius, 2000). South African wines therefore are more likely to contain species of *Lactobacillus* and *Pediococcus* as the pH 's are higher. Inoculation with starter cultures

and the concomitant immediate onset of MLF are beneficial to avoid spoilage of high pH wines, which include South African red wines.

pH, ethanol and temperature have been found to work synergistically. At pH 2.9-3.0 growth is possible for LAB but extremely slow. At pH > 3.5 growth is much quicker when the alcohol levels are less than 13% v/v and a temperature between 19-20˚C is maintained. Growth conditions that do not support the growth of LAB in wine include a pH < 3.0, ethanol levels > 14% v/v and temperatures below 17˚C (as cited by Lonvaud-Funel, 1995). The optimal pH for malolactic activity for *O. oeni* is in the vicinity of pH 3.5-4.0 (Davis *et al*., 1986).

### 2.4.2 Temperature

Temperature is a well known catalyser for chemical and biochemical reactions. In a laboratory culture medium it was found that LAB grew in a temperature range between 15 and 45˚C, with optimal growth occurring between 20 and 37˚C. For *O. oeni* the optimal temperature range was 27-30˚C in laboratory medium but was 20-23˚C in wine (due to the alcohol in the medium). ~~This optimum temperature will decrease when the alcohol content of wine ranges between 13-14%.~~ Virtually no growth will occur at 14-15˚C. The ideal temperature for malic acid degradation is approximately 20˚C. At temperatures above 25˚C and below 18˚C, MLF times are delayed (Ribereau-Gayon *et al*., 1998).

Guzzo *et al*. (1994) found that when *O. oeni* was pre-incubated at 42˚C, the survival and ability of the strain to perform MLF was enhanced. Incubation at this temperature induces the formation of stress proteins (Guzzo *et al*, 1997).

### 2.4.3 Ethanol

Ethanol and temperature have antagonistic effects on the growth of LAB. In wines with high ethanol concentrations, the optimal growth temperature of the LAB will decrease. Ethanol tolerance is decreased at higher temperatures (Henick-Kling, 1993). The ethanol levels found in wine (8-12% v/v) are not inhibitory for malolactic activity (Capucho and~~&~~ San Romao, 1994). The growth rate decrease linearly with the increase in the alcohol level and 14% v/v alcohol is the upper limit for growth of most of the strains of LAB (Davis *et al*., 1988; Henick-Kling, 1993). ~~At 25˚C growth will be completely inhibited in the presence of 10 to 14% v/v alcohol. With the latter alcohol levels, optimum growth/yield will~~

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~~be in the vicinity of 18-20˚C, compared to 30˚C when ethanol levels are 0-4% v/v (Henick-Kling, 1993). Ethanol and temperature have a greater affect on growth rate than biomass yield.~~

The level of ethanol tolerance differs between strains and is also dependant on the amount of nitrogen in the medium and the pH (Britz and Tracey, 1990). Ribereau-Gayon *et al*. (1975) found that cocci are more sensitive to alcohol than other LAB species and Davis *et al.* (1988) went further to say that *Lactobacillus* spp. and *Pediococcus* spp. are generally more tolerant to ethanol than *O*. *oeni*.

The cell's ability to tolerate high ethanol levels will primarily be located in the cell membrane, with lipids as the major target area (Jones, 1989). Ethanol-induced changes in the lipid composition of the cell membrane have been described for *O. oeni* (Tracey and Britz, 1989a; Garbay *et al*., 1995) and other LAB. In the presence of alcohol, the cell membrane fluidity is enhanced in *O*. *oeni* (Couto *et al*., 1996; Tourdot-Marechal *et al*., 2000; Teixeira *et al*., 2002). Tourdot-Marechal *et al*. (2000) found that the increase of lactic acid in the membrane is possibly involved in protecting the cell from high ethanol levels (> 8% v/v). Lactobacillic acid is a ring containing fatty acid produced during late exponential to stationary phase growth and is formed by conversion of the unsaturated position of *cis*-vaccenic acid to a cyclo-propane ring. Teixeira *et al*. (2002) showed that ethanol levels > 8% increased the permeability of the cell membrane of resting cells, but not when cells was grown in these alcohol conditions. The amount of protein in the latter was found to be lower. Guzzo *et al*. (1997, 2000), Tourdot-Marechal *et al*. (2000) and Texiera *et al*. (2002) all showed that the synthesis of low-molecular weight stress proteins is induced and may also be involved in the adaptation of cells.

Thus, the resistance of *O*. *oeni* cells to alcohol involves an array of parameters that include media composition, pH, temperature and the severity and duration of the shock that is exerted on the cells.

### 2.4.4 Sulphur dioxide

#### 2.4.4.1 Sulphur dioxide in wine

Sulphur dioxide ($SO_2$) is added to wine and acts as an antioxidant and prevents the growth of detrimental micro-organisms (Amerine *et al*., 1980; Facio and Warner, 1990). In wine, $SO_2$ ~~sulphur dioxide ($SO_2$)~~ exists in equilibrium between its free and bound form. The

free form is mainly responsible for the antimicrobial and anti-oxidative activity and consists of a molecular $SO_2$, bisulphite and sulphite component (Du Toit *et al*., 2005)

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The levels at which sulphur dioxide will influence the development of LAB, and consequently MLF, are: total concentration between 100 and150 ppm $SO_2$ and 1-10 ppm free $SO_2$ (Wibowo *et al*., 1985).

#### 2.4.4.2 Effect of sulphur dioxide

Carreté *et al*. (2002) showed that the $SO_2$ concentration should not be too high in wine because in can inhibit the growth of LAB during MLF. Constanti *et al*. (1998) even suggest that the use of $SO_2$ could be eliminated if the yeast selected to conduct AF suppresses bacterial growth.

Liu and Gallander (1983) found that $SO_2$ levels affect the growth behaviour of malolactic bacteria and the rate of MLF and that it is strongly related to the initial pH of the wine. Kunkee (1968) also found that when must has a low pH and is treated with $SO_2$, the inhibitory effect that occurs is related to the influence of the pH on the metabolic rate of LAB as well as to the antimicrobial activity of $SO_2$. Low pH and high $SO_2$ levels greatly reduced survival of the inoculated bacteria and thus the MLF rate. They also found that for the same $SO_2$ levels, the rate of MLF was similar for pH 3.5 and 3.7 but slower in pH 3.3. For any given pH the lowest $SO_2$ level resulted into the fastest MLF rate. After inoculation with the MLF starter culture the bacterial numbers decreased until week three after inoculation for all the treatments but the lowest population count was found for pH 3.3. Therefore, the initial treatment of must with $SO_2$ is an important factor to be considered when using pure culture inoculation to induce MLF.

In addition to adding sulphur dioxide to grape must, it can also be formed by the yeast during alcoholic fermentation. Fuster *et al*. (2002) showed that some yeast strains, especially ones with low nutritional demands, can favour the onset of MLF while other yeast inhibits LAB and MLF. This inhibition is a result of the yeast generating compounds that are toxic to LAB like $SO_2$, higher ethanol and fatty acids (Guilloux-Benatier *et al*., 1998; Henick-Kling and Park, 1994; Lonvaud-Funel *et al*., 1988). Since the type and amount of fatty acids and other macromolecules that are released by yeast into the wine media is strain dependent, the evolution of LAB and subsequently MLF are also dependent on yeast strain (Fornachon, 1968; Huang *et al*., 1996; King and Beelman, 1986; Larsen *et al*., 2003).

$SO_2$ could be a health risk for sulphite-sensitive individuals, there is an ever-increasing consumer demand to reduce $SO_2$ levels in foods and beverages. Studies combining the use of $SO_2$ with other compounds that reduces oxidation and bacterial growth

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~~**2.4.5Phenolic compounds**~~

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~~Phenolic compounds can broadly be divided in into two groups namely, the non-flavonoids and the flavonoids. The non-flavonoids are subdivided into the phenolic acids and their derivates namely *p*-hydroxy-benzoic acid, cinnamic acid derivatives (**Figure. 2.43**) and other compounds which include *m*-cresol and tyrosol. These compounds occur at approximately 100-200 mg/L in red wine and 10-20 mg/L in white wine (Ribéreau-Gayon *et al*., 1998). Mostly they occur as glucose esters.~~

~~The flavonoids are divided into flavonols, flavan-3-oles, flavan-3,4-dioles and anthocyanins. The flavonols are flavonoid structures that are esterified with glucose at position 3. Production of flavonols starts in the berry as soon as it has been exposed to sunlight and resides in the skin of the grape. They protect the berry against UV light (Sweeny *et al*., 1981) and are yellow in colour. Examples of important flavonols are Kaempherol and Quercetin (**Figure 2.54**) (Ribéreau-Gayon *et al*., 1998).~~

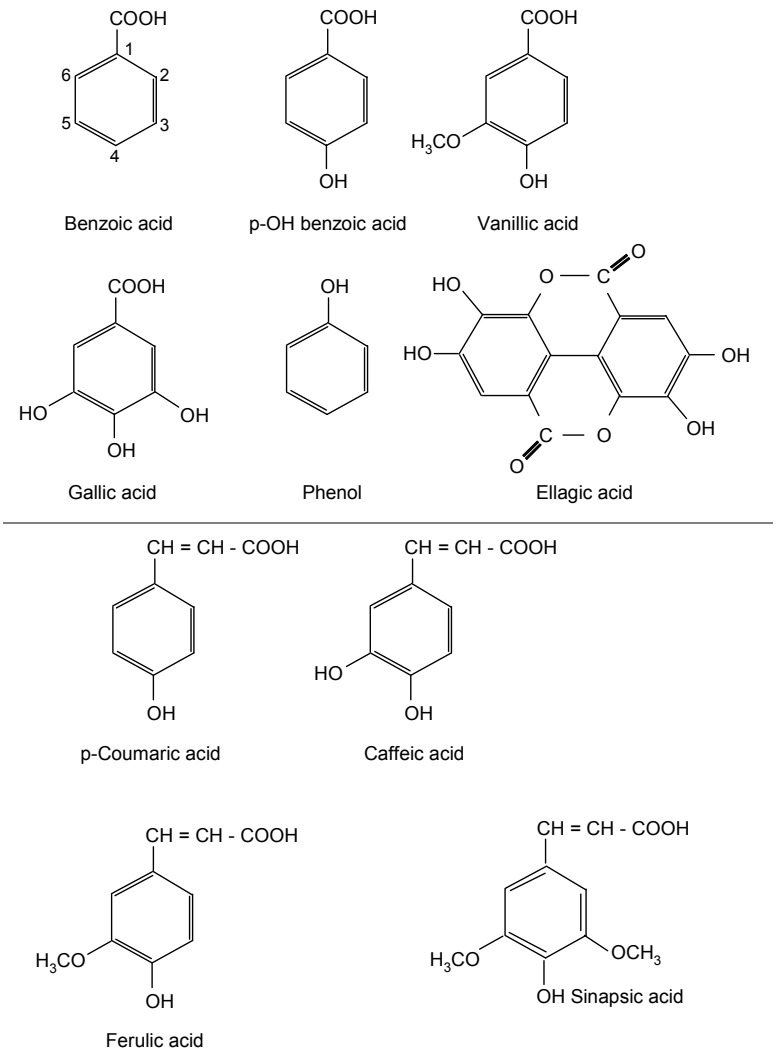


**Figure 2.43** Different non-flavonoids of grapes and wine

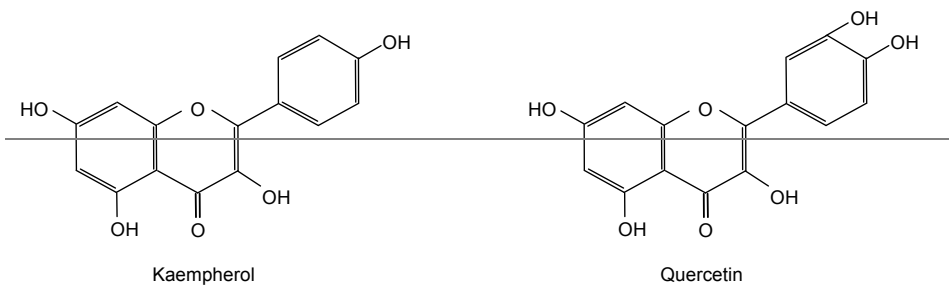


**Figure 2.54** Flavonols of wine Kaempherol and Quercetin

Flavan-3-ole, also called catechin, is characterised by an OH group on position 3 of a saturated C ring (**Figure 2.65**). The forms of catechin and epicatechin that naturally occur in grapes are (+)-catechin and (-)-epicatechin. These compounds can also occur as dimers, oligomers and polymers. They form condensed tannins and play and important role in the taste of wine. Singleton and Esau (1969) found that catechin and epicatechin concentration in white wines range from 10-50 mg/L and 200 mg/L in red wines.

Flavan-3,4-dioles are also known as leucoanthocyanidins. These compounds are characterised by OH bonds in position 3 and 4 on the C-ring of the flavonoid structure (**Figure 2.65**). When these compounds polymerise they form their corresponding condensed tannins (Ribéreau-Gayon *et al*., 1985; Zoecklein, *et al*., 1995). These compounds are usually present in the form of oligomers for example, leucocyanidin, procyanidin, leucodelphinidin and prodelphinidin.

Anthocyanins are important for the colour of red wine (**Figure 2.76**). Anthocyanins consist of a anthocyanidin esterified to glucose. These compounds play a major role in the oxidation sensitivity of must and wine, and reside in the skins of the berries. Anthocyanins are present at a level between 100-1500 mg/L in wine (Monangas *et al*., 2005; Ribéreau-Gayon *et al*., 1998; Somers, 1971).

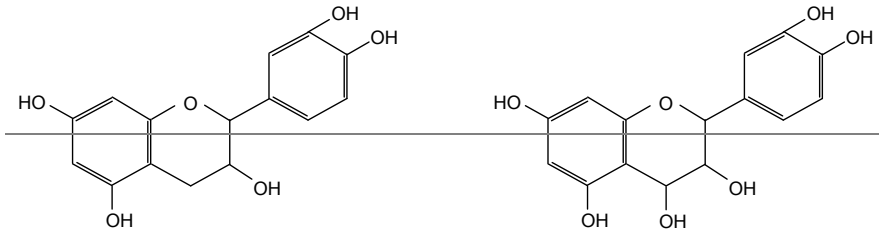


**Figure 2.65** Flavan-3-ole and Flavan-3,4-diole

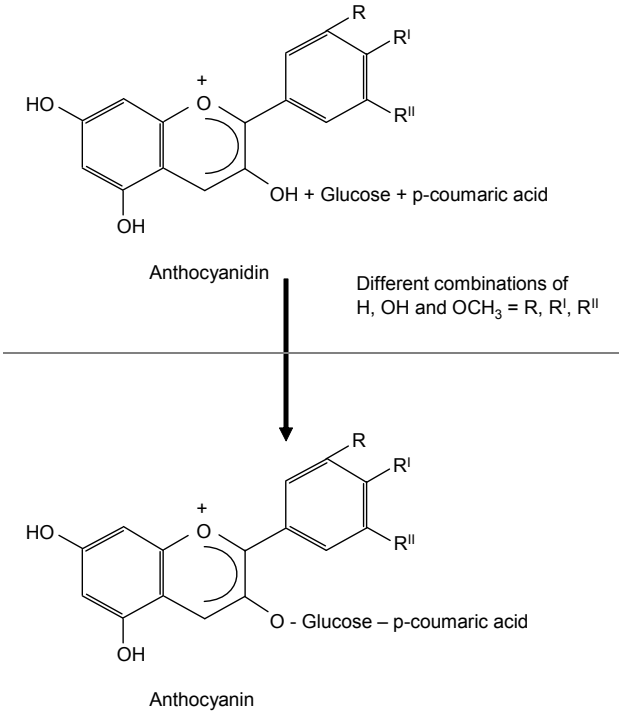


**Figure 2.76** Anthocyanin structure

**2.4.5.1The influence of phenolic compounds on LAB and MLF**

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During winemaking, phenolic compounds are extracted whilst the must is in contact with the grape skins. As early as 1970, Beelman and Gallander conducted an experiment where MLF was induced in grape must prepared by cold pressing, hot pressing and fermentation on the skins for 1, 3 and 5 days before pressing. The results revealed that fermentation on the skins had a profound effect on MLF. MLF was completed only in the 5 day treatment of fermentation on the skins. They stated that skin contact must have stimulated the growth of the MLF bacteria.

Later studies were mostly done with the phenolic acids and their potential influence on MLF. Vivas *et al*. (1997) found that gallic acid enhanced cell growth and rate of MLF of *O. oeni*, whilst vanillic acid was slightly inhibiting. Another study by Alberto *et al*. (2001) showed that gallic acid activated the rate of glucose and fructose utilization and that the gallic acid was consumed from the beginning of *L. hilgardii* growth. Therefore gallic acid could potentially increase the formation of spoilage compounds in the presence of *L. hilgardii*. Compos *et al*. (2002) monitored an ethanol containing medium supplemented with varying concentrations of hydroxybenzoic acids and hydroxycinnamic acids. It was found that the hydroxycinnamic acid was more inhibitory to *O. oeni* than the hydroxybenzoic acids (gallic and vanillic acid). The hydroxycinnamic acids (caffeic and ferulic acid), were more beneficial to the growth of *L. hilgardii*. *p*-Coumaric acid had the strongest inhibitory effect on the growth and survival of both bacterial species. Hydroxycinnamic acids have also been found to have an inhibitory effect on *O. oeni* at high concentrations (Reguant *et al*., 2000). They also showed that catechin and quercetin (flavonoids) stimulated MLF but delayed or inhibited the formation of acetic acid from citric acid. This could suppress the increase in volatile acidity (VA) and therefore control MLF better. Catechin also stimulated MLF (measured as malic acid consumption) for *L. hilgardii* (Alberto *et al*., 2001). Vivas *et al*. (1997) also looked at the effect of anthocyanins on the growth of *LO. oenios* and the rate of malic acid degradation and found that it activated both processes.

It is important to note that in most of these studies, the phenolic compounds were used at concentrations higher than what would naturally occur in wine. Another important factor to mention is that synthetic media were used in most cases. This eliminated other interactions that could possibly have occurred in a complex medium like wine.

## 2.5 HEALTH RISKS ASSOCIATED WITH SPONTANEOUS MALOLACTIC FERMENTATION

### 2.5.1 Biogenic amines in general

Biogenic amines (BA) are toxic substances that have deleterious effects on the health of humans (Shalaby, 1996). These substances can be found in various fermented foods and beverages such as fish, cheese, beer and meat products (Stratton *et al*., 1991; Shalaby, 1996). BA's are undesirable in all food and beverage products in which they occur. Symptoms that are experienced as a result of the ingestion of BA's are headaches, respiratory distress, heart palpitations, hyper- or hypotension, and several allergenic disorders (Sillo Santos, 1996).

The extents to which BA's can be toxic to humans vary due to at least two important factors. The first being the detoxifying effect of the human body on amines and the second, the inhibition of important enzymes that play a role in the formation of BA's, through various drugs and ethanol. Thus when the toxic effects of BA's are to be estimated the following must be taken into account: the quantity of food, the concentration of total BA's, and the consumption of ethanol and drugs (Lonvaud-Funel, 2001).

Amines are formed by LAB during fermentation of foods and beverages by amino acid decarboxylation. Various LAB genera are able to perform this reaction, which is thought to favour growth in acidic media.

With consumers demanding healthier and better controlled production of food products, there is a renewed interest surrounding the study of biogenic amines of wine. The best developed method for the determination of BA's in especially wine, is high performance liquid chromatography (Rollan *et al*., 1995).

#### 2.5.1.1 Biogenic amines of wine

The major BA's in wine are histamine, tyramine, putrescine and cadaverine resulting from the decarboxylation of the the corresponding amino acids namely, histidine, tyrosine, ornithine and lysine. Histamine is the most toxic amine and it can be potentiated by other amines (Chu and Bejdanes, 1981). The levels of BA's in wine are much lower than the levels at which they occur in other fermented products but the presence of other substrates such as ethanol, 1-methylhistamine, methylamine, ethylamine, tryptamine,

2-phenylethylamine, tyramine, putrescine, cedaverine and spermidine may increase the toxicity of histamine and exceed the limits for sensitive people (Guerrini *et al*., 2002). Putrescine is the amine that is generally found in the highest concentration in wine (Soufleros *et al*., 1998) and is also known as the most effective potential activator of histamine toxicity to humans (Taylor, 1986). Putrescine and cadeverine are also potential precursors of carcinogenic nitrosamines (Bover-Cid and Holzapfel, 1999).

~~Since LAB are responsible for producing BA's, it is assumed that all LAB contain decarboxylase and the transport system (the enzymatic equipment to allow the reaction).~~

Grape variety and viticultural practices influence the constitution of the grape must (Soufleros *et al*., 1998), which will undergo AF. Therefore, the levels of BA's present in wine will reflect a combination of factors which include the micro-flora present, the constitution of the grape must and yeast metabolism during AF**.** LAB only develops after AF in wine, which means that the constitution of the must will have changed in terms of its nitrogen composition. Another wine process that will influence the level of amines is extended lees contact, where various peptides and free amino acids are released into the wine that could be utilised by LAB. It is also important to note that the ability of bacteria to decarboxylate amino acids is strain dependent (Coton *et al*., 1998). Aside from the amount of precursor and the strain of LAB present in the wine, pH are another im~~The most important factor influencing~~ portant factor influencing the production of BA's is pH. Higher pH's generally result in higher BA levels in wine (Lonvaud-Funel and Joyeux, 1994) as a high pH will allow for the development a more diverse range of micro-flora. This effect of pH is illustrated by the observation that in white wines, which generally have a lower pH than red wines, the concentration of BA's is lower (Lonvaud-Funel, 2001).

It has also been found that BA levels not only increase during MLF but also during ageing (Lonvaud-Funel, 2001). This was the case with Chardonnay and Pinot noir wines studied by Garbaux and Monany (2000)~~as cited by Lonvaud-Funel, 2001)~~. They also showed that the most active phase was between the fourth and eight month after MLF. As wine is treated with $SO_2$ after MLF, these results show that not all biochemical reactions mediated by bacteria are effectively inhibited. Sulphur dioxide is especially less effective in red wines with a high pH.

#### 2.5.1.2 *Oenococcus oeni* and biogenic amines

The first studies on biogenic amines showed higher levels occurring in European, American and South African red wines than in white wines (Zee *et al*., 1983; Cilliers and Van Wyk, 1985). Histamine is the most important BA that occurs ~~most frequently~~ in wine. Some authors consider the increase of histamine levels to be as a result of MLF, whilst other authors do not connect the two. Unfavourable LAB, *Pediococcus* spp., has always been differentiated from the favourable LAB, *O. oeni* and the prior mentioned was also solely held responsible for histamine production. ~~*Pediococcus* spp have been held responsible for histamine production for a long time and even still today (as cited by Lonvaud-Funel, 2001)~~.

Lonvaud-Funel and Joyeux (1994) extensively studied the micro-flora of wine containing BA's after MLF. They isolated strains of *O. oeni* that tested positive for histamine production. Landete *et al*. (2005) used a qualitative method based on pH changes in a plate assay to detect wine strains capable of producing high levels of histamine. They found that *O oeni* showed the highest frequency to produce BA's whilst *Lactobacillus* and *Pediococcus* spp. produced the highest concentration of BA's. Guerrini *et al*. (2002) also found that *O. oeni* could contribute significantly to the overall biogenic amine concentration in wine. Of the 44 strains tested, 60% produced a level of histamine between 1.0-33 mg/L and about 16% produced additional putrescine and cadeverine. In a study by Konings *et al*. (1997) it became clear that the production of histamine is enhanced in poor growth conditions for example, when fermentable substrates like sugar and malic acid are limited.

*O. oeni* has also been found to produce putrescine. Mangani *et al*. (2005) found that *O. oeni* can produce this BA from ornithine as well as arginine.

### 2.5.2 Ethyl carbamate

Ethyl carbamate (EC) is found in wine amongst other foods and beverages and is an animal carcinogen (Ough, 1976). It is formed through the chemical reaction of ethanol and an EC precursor, such as citrulline, urea or carbamyl phosphate (Ough *et al*., 1988). Citrulline is an intermediate in the degradation of arginine by wine LAB. A correlation has been found between the excretion of citrulline and the formation of EC during the degradation of arginine by the wine LAB *O. oeni* and *L. buchneri* (Liu *et al*., 1994). Another precursor of EC is carbamyl phosphate. Some LAB can synthesize carbamyl phosphate from glutamine and bicarbonate and ATP (Nicoloff *et al*., 2001).

## 2.6 CONCLUSION

MLF is an important process during winemaking and the outcome of this process plays an important role on the over-all quality of wine (especially red wine). *O. oeni* is the major LAB that conducts this process because of its greater tolerance of acidic wine conditions and the favourable attributes it makes to wine aroma. Nevertheless, various factors can influence this process either positively or negatively and to a larger and lesser extent. In an effort to control MLF and the LAB that conducts the process, factors that could negatively influence the process must be managed. One possibility to reach this goal was the development of the MLF starter culture. Starter cultures proposed a sure and successful MLF if wine properties are maintained within certain limits. Overall, for desired growth and MLF by *O. oeni* it is optimum to have a low pH (<3.5), a temperature of 20-25ºC, small amounts of $SO_2$ (< 10 mg/L free), no or little pesticide residues and an alcohol level below 13.5%. Starter cultures have been constantly improved with regards to their tolerance to winemaking practices, wine conditions and rate of MLF. Research regarding factors that could enhance this process are not only beneficial to the production process of wines but can contribute to the quality of the end product.

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# 3. RESEARCH RESULTS

## The stretching of malolactic fermentation starter cultures in high pH red wines

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**ABSTRACT**

This study evaluated stretching of malolactic fermentation (MLF) starter cultures, methods regularly used in practice, in high pH South African red wines (Merlot, Pinotage and Cabernet Sauvignon). Stretching of MLF starter cultures refers to using less of the recommended dosage of MLF starter cultures or the re-use of starter cultures. Stretching may result in economical advantages but is also thought to be easy to apply on high pH wines, which are more favourable conditions for lactic acid bacteria (LAB) to exist in. The objective of this study therefore was to evaluate the difference in MLF rate between the different stretching treatments as well as to investigate the influence of the naturally occurring LAB within the different stretching treatments. The control for this study was a 100% w/v inoculation with a starter culture as prescribed by the suppliers. The stretching treatments included a 50% w/v and 25% w/v of the dosage inoculation as well as a mothertank and lees inoculation. The parameters monitored included weekly monitoring of the malic acid degradation, enumeration of the wine LAB on selective media and biogenic amine (BA) levels at the end of MLF. The results showed that the mother tank and lees treatments resulted in an increased rate of MLF, with the 25% w/v inoculation having the slowest rate in most instances. The enumeration data showed that at the end of MLF *Oenococcus oeni* was the LAB species most likely present. No definite trends for BA's were found between the treatments.

## 3.1 INTRODUCTION

Malolactic fermentation (MLF) in wine is a secondary fermentation that usually occurs at the end of alcoholic fermentation (AF). MLF refers to the biological process of wine deacidification in which the dicarboxylic L-malic acid is converted to the monocarboxylic L-lactic acid and carbon dioxide (Davis *et al*., 1985).

Besides the decrease in acidity, it also improves the microbiological stability and the organoleptic characteristics of wines (Davis *et al*. 1988; Kunkee, 1991). These above-mentioned organoleptic changes are due to secondary metabolisms (Lonvaud-Funel, 1999), such as the metabolism of carbohydrates and amino acids. The most important compounds apart from lactic acid that is formed are acetic acid, diacetyl (buttery flavour), acetoin, acetaldehyde, 2,3-butanediol, 2-acetolactate, 2-acetohydroxybutyrate, ethyl acetate and ethyl lactate (Fornachon and Lloyd, 1965; Henick-Kling *et al*., 1994; Kandler, 1983; Mascarenhas, 1984).

Malolactic fermentation is conducted by lactic acid bacteria (LAB) that contains the malolactic enzyme (MLE). *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* are all genera of LAB that are present in wine. Towards the end of AF spontaneous MLF is mainly conducted by *O. oeni* (Van Vuuren and Dicks, 1993), a species formerly known as *Leuconostoc oenos* (Dicks *et al*., 1995).

Spontaneous MLF may occur any time during or several months after the completion of AF. At the end of AF the wine is not protected with sulphur dioxide and it is therefore important that MLF commences as soon after AF as possible. To resolve this issue, MLF starter cultures are used to induce MLF.

MLF starter cultures consist of pure culture *O. oeni* that was isolated from wine. Commercial starter cultures (freeze dried) are usually inoculated into the wine after completion of AF at a cell concentration of superior to $5 \times 10^6$ cfu/mL.

The stretching of starter cultures imply using less than the recommended dosage, but can also imply re-use of commercial starter cultures as in the case of mothertank inoculation as well as inoculation from the lees of wines that have finished MLF. Stretching of MLF starter cultures could lead to economical advantages, but the success of the starter cultures as well as the role of the natural occurring LAB have not been determined. Earlier studies showed that when commercial starter cultures are directly inoculated into wine in the freeze dried form a decrease of down to 4 log units could be observed (Krieger *et al*., 1990). Other authors obtained similar results with a reduction of

3-4 log cycles after direct inoculation with freeze dried cultures (Fugelsang and Zoecklein, 1993; Henick-Kling, 1993), whilst Nielsen *et al*. (1996) demonstrated the 100% survival of a freeze dried starter culture after direct inoculation into wine. Inoculation data as was found by the above-mentioned authors underline the sensitivity of different strains of LAB to the wine matrix.

Therefore the stretching of commercial starter cultures could have a detrimental effect on the performance of bacteria during MLF. The pH of South African wine is generally higher (3.4-4.0) than cool climate regions, due to the warmer climate. Higher pH and lower sulphur dioxide levels could lead to unwanted LAB and even acetic acid bacteria (AAB) development in wine.

Biogenic amines (BA) are toxic substances that have deleterious effects on the health of humans (Shalaby, 1996). Amines are formed by LAB during fermentation of foods and beverages by amino acid decarboxylation. The most important factor affecting bacterial strain capability to produce BA's is pH. A high pH generally produces a higher BA level in wine (Lonvaud-Funel and Joyeux, 1994).

The specific aims of the study were to evaluate the difference in MLF rate, to investigate the influence of the natural microflora and to determine the levels of biogenic amines that wereas formed during the different stretching treatments in high pH South African red wines.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Small-scale fermentation

The experiments were performed during the 2005 and 2006 harvesting season on three red wine cultivars. Merlot, Pinotage, and Cabernet Sauvignon grapes from the Stellenbosch (South Africa) wine region with high pH's (3.7-4.0) were used.

The grapes were crushed and 30 mg/L sulphur dioxide was added to the must. The juice was analysed for pH, titratable acidity (TA) and ºBrix. The juice was fermented with WE 372 (30 g/hL) (Anchor Yeast Biotechnologies, South Africa) in 250 L open fermenters on the skins. Punch downs were done twice daily throughout the AF. When the residual sugar concentration was less than 5 g/L the skins were pressed and transferred into stainless steel tanks until completion of AF. After this the wines were then divided for the different treatments. Sample bottles consisted of 4.5 L glass bottles closed with rubber stoppers with S-shaped airlocks filled with water. MLF was inoculated into these

containers for the different treatments. These sample bottles were kept at a temperature of 23˚C for the duration of MLF. Each treatment was carried out in triplicate for this study.

The experiment consisted of a control and four treatments. The control was a 100% w/v inoculation with a commercial MLF starter culture as prescribed by the manufacturers (1.5 g/250 L or > 5 X $10^6$ cfu/mL). Treatment 1 refers to inoculation of a commercial MLF starter culture at 50% w/v of the recommended dosage. Treatment 2 was inoculated at 25% w/v of the recommended rate. Treatment 3 was inoculated for MLF by using a mother tank (MT) inoculation. Inoculation~~ng~~ of new wine proceeded at a rate of 10% v/v, after 10% of the malic acid was degraded in the mother tank wine ~~at a 10% v/v rate~~. Treatment 4 consisted of inoculating new wine with the lees (L) from the control wine which completed MLF.

This layout was followed for all the cultivars in 2005 and 2006 except for the Merlot 2006 where additional treatments were included. These treatments consisted of a control, treatment 1 and treatment 2 with Velcorin (V) added to each. The Velcorin (250 ppm) (Bayer, Germany) was added to these wines 48 hours before inoculating with the MLF starter cultures to sterilise the wine.

~~All treatments were performed in triplicate.~~

All treatments were conducted with the following freeze-dried starter cultures, Viniflora®oenos, Viniflora®CH16 and Viniflora®CH35 (ChrHansen, Denmark). These cultures are produced to inoculate directly into the wine without an activation step. Each starter culture was isolated because of a unique characteristic it consists of according to the supplier. For instance Viniflora®oenos gives a clean and classic flavour profile, whilst Viniflora®CH16 has an excellent tolerance to high alcohol levels and Viniflora®CH35 is a strong fermenter in harsh white wines. All three cultures do not produce BA's according to the producer. All treatments were performed in triplicate.

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### 3.2.2 Monitoring of wine parameters by FT-IR

L-Malic acid was measured weekly from inoculation of the MLF starter culture using FT-IR spectroscopy (Foss Grape scan). Samples were filtered with a Filtration Unit (type 79500, FOSS Electric, Denmark) connected to a vacuum pump. The filter unit uses filter paper circles graded at 20 – 25 µm with diameter 185 mm (Schleicher & Schnell, reference number 10312714). The filtered musts were used for FT-IR spectral measurements. A Winescan FT120 equipped with a purpose built Michelson interferometer was used to

generate the FT-IR spectra (FOSS Electric A/S, Hillerød, Denmark). Instrument settings included: cell path length of 37 µm, sample temperature set to 40°C, and sample volume of 7 – 8 ml. The sample is pumped through the heat exchanger and the $CaF_2$-lined cuvette. Samples are scanned from 5011 to 926 $cm^{-1}$ at 4 $cm^{-1}$ interval.

Global calibrations were used for the FT-IR spectroscopic analysis, although further calibration will be needed for South African conditions in terms of malic acid determinations.

Other analysis that was done with FT-IR technology is the monitoring of routine wine parameters (pH, Total acidity (TA), Volatile acidity (VA), Lactic acid, Ethanol, Glucose, Fructose and Glycerol) at different stages of the winemaking process. These stages were the juice after crushing and the wine after AF, during MLF up to the end of MLF.

### 3.2.3 Media and culture conditions for enumeration of wine LAB

The samples were enumerated on MRS agar (Biolab, Merck, South Africa) enriched with 20% apple juice (containing no preservatives) (MRSA) (pH 5.2). The addition of apple juice and lowering of the pH stimulates the growth of to select for *O. oeni*. Normal De Man, Rogosa and Sharpe (MRS) (Biolab, Merck, South Africa) agar was also used for the enumeration of potential naturally occurring LAB. Both MRSA and MRS contained 100 mg/L Actistab (50% glucose, 50% natamycin, Gist-brocades, France, S.A.), dissolved in methanol, for the inhibition of moulds and yeast. Kanamycin sulphate ($C_{18}H_{36}N_4O_{11}$ X $H_2SO_4$, Roche Diagnostics, dissolved in sterile distilled water) at 25 mg/L were used for the inhibition of AAB. The MRSA and MRS was incubated under facultative anaerobic conditions at 30˚C (Anerogen, Oxoid) in a rectangular anaerobic jar (Davies Diagnostics (Pty) Ltd).

Plating for this study was done on three pivotal stages of the winemaking process. Firstly, after crushing the juice, then after AF and also when MLF had been completed. The samples were diluted within a range of $10^{-1}$-$10^{-6}$ using 1 mL of sample and test tubes filled with 9 mL distilled water (autoclaved).

### 3.2.4 Biogenic amine analysis

After MLF the biogenic amine level in the wines were measured for the Merlot and Cabernet 2005 samples as well as for the Cabernet 2006 samples, using high

performance liquid chromatography (HPLC) at Distell, South Africa (Alberto *et al.,* 2002). Histamine, Tyramine, Putrescine and Cadaverine were determined. The samples were filtered with a 0.22µm filter and diluted 10 times for these analyses.

## 3.3 RESULTS AND DISCUSSION

### 3.3.1 Chemical properties of the wines

After alcoholic fermentation the highest wine pH was found in the Pinotage 2006 (4.2) followed by the Cabernet Sauvignon 2005 (4.0) (**Table 3.1**). The Pinotage 2005, Merlot and Cabernet Sauvignon 2006 had the same pH of 3.85, whilst the Merlot 2005 had the lowest pH of 3.77 (**Table 3.1**). Ribereau-Gayon *et al*. (1998) have shown that the microflora of wine is more abundant and also more diverse in high pH conditions when compared to more acidic wines. The ethanol concentration ranged from 12.3%–14.7% (**Table 3.1**). High ethanol levels can be inhibitory for the growth of LAB in wine. It has been shown that the growth rate decrease linearly with the increase in the alcohol level and 14% v/v alcohol is the upper limit for growth of most of the strains of LAB (Davis *et al*., 1988; Henick-Kling, 1993).

**Table 3.1** pH, TA, VA and ethanol levels after AF for the Merlot, Pinotage and Cabernet Sauvignon used for the stretching experiments in the 2005 and 2006 harvesting seasons.

| | 2005 | | | 2006 | | |
|---|---|---|---|---|---|---|
| | Merlot | Pinotage | Cabernet Sauvignon | Merlot | Pinotage | Cabernet Sauvignon |
| pH | 3.77 | 3.85 | 4.00 | 3.85 | 4.20 | 3.85 |
| TA (g/L) | 6.75 | 5.20 | 5.65 | 6.25 | 5.63 | 6.60 |
| VA (g/L) | 0.27 | 0.57 | 0.40 | 0.05 | 0.38 | 0.22 |
| Ethanol (%v/v) | 14.50 | 13.200 | 12.32 | 14.15 | 14.50 | 14.75 |

### 3.3.2 Merlot 2005 and 2006

For the Merlot 2005 inoculated with Viniflora®oenos the initial malic acid degradation was the fastest in the MT treatment. The malic acid was reduced with 68% at week 1, in comparison to the other treatments that only showed a reduction of between 25%-50% (**Figure 3.1**). The Control (100% inoculation) had the second highest degradation level of

malic acid (50%), followed by the Lees treatment (44%), the 50% treatment (28%) and the 25% treatment (25%).

During week 2 the malic acid reduction of the MT, 50% and 25% was more or less the same level of 80%, followed by the Control and Lees treatment with a reduction percentage of 70%.

At week 3 the malic acid reduction percentages reached more or less the same level for all the treatments, which continued to week 4 with the exception of the MT treatment for which no malic acid was detected. The MT treatment therefore finished MLF within 3 weeks (therefore in week 4 no malic acid was detected for the MT, **Figure 3.1**).

Week 1 and week 2 was the points in time at which the treatments showed the greatest difference in malic acid degradation. Malic acid degradation were faster in the 100% inoculation than the 50%, 25% and L treatments for the first week, where after the 50% and 25% treatments reached the same level and these treatments finished after 4 weeks. The L treatments had a faster rate than the 50% and 25% over the first week of MLF after which the rate decreased and took a week longer than the other treatments to finish MLF in the end. The initial higher rate of malic acid degradation could be due to the reason that for the L treatment the starter culture was already adapted to the wine conditions in which it was inoculated. Another reason could be that although the initial cell numbers were higher the growth phase of the cells were at the end of stationary phase and when inoculated into the wine a large percentage died-off and therefore a decrease in MLF rate were observed.

For the Merlot 2005 wine inoculated with Viniflora®CH16 and Viniflora®CH35 (**Figures 3.2** and **3.3** respectively) the pattern of malic acid reduction was the same as for the inoculations with Viniflora®Oenos, except in week 1 (for Viniflora®CH16) and for the MT treatments (Viniflora®CH16 and C35). For the Viniflora®CH16 inoculation in week 1 the order of malic acid reduction was MT>L>C>50%>25% (in the other two inoculations the Control had a higher reduction than the Lees treatment at week 1). For both Viniflora®CH16 and CH35 malic acid was still detected in the MT treatment at week 4.

The cell numbers obtained at the end of MLF for all the treatments and starter cultures used did not show significant differences (**Figure 3.4**). It varied between $5 \times 10^6$ and $1.8 \times 10^7$ cfu/mL. The MT treatments (with all three starter cultures) had higher cell numbers than the other treatments, which could explain the faster MLF rate. In future to clarify the results cell numbers have to be determined during the course of MLF. In the MT

and L treatments it will be important to determine the contribution of the natural LAB to MLF.

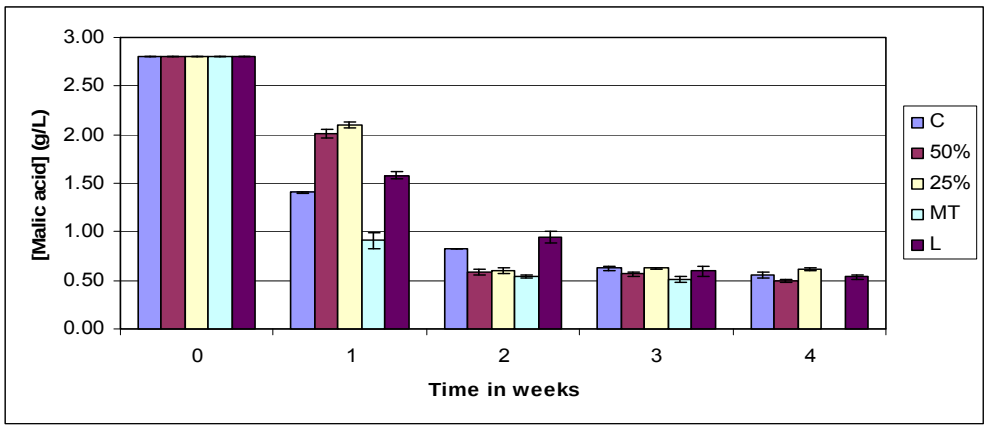


**Figure 3.1** Degradation of malic acid during MLF in Merlot (2005) where different inoculation treatments were evaluated. All treatments were inoculated with Viniflora–®oenos. C represent the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represent the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatments that were inoculated from the control samples after MLF was finished. Error bars indicate the standard deviation for the three repeats.

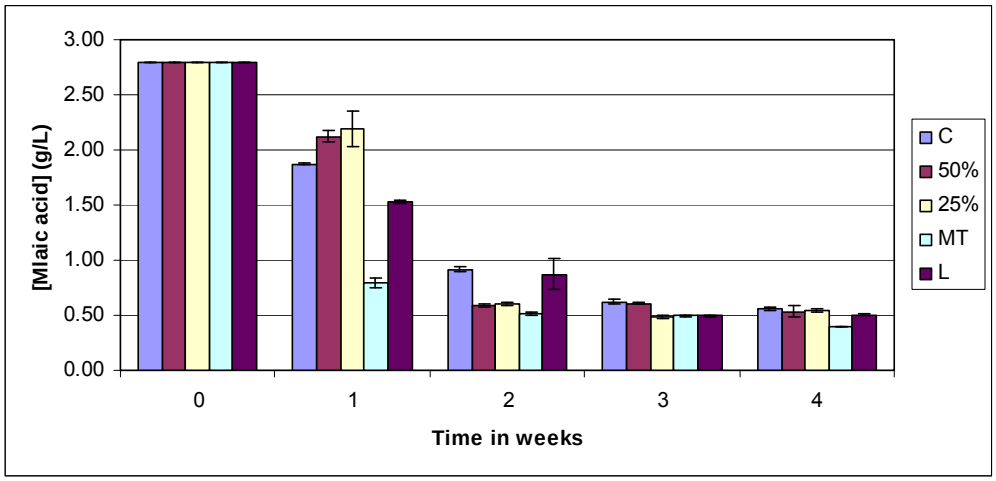


**Figure 3.2** Degradation of malic acid during MLF in Merlot (2005) where different inoculation treatments were evaluated. All treatments were inoculated with Viniflora®CH16. C represents the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represent the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatments that were inoculated from the control samples after MLF have finished. Error bars indicate the standard deviation for three repeats.

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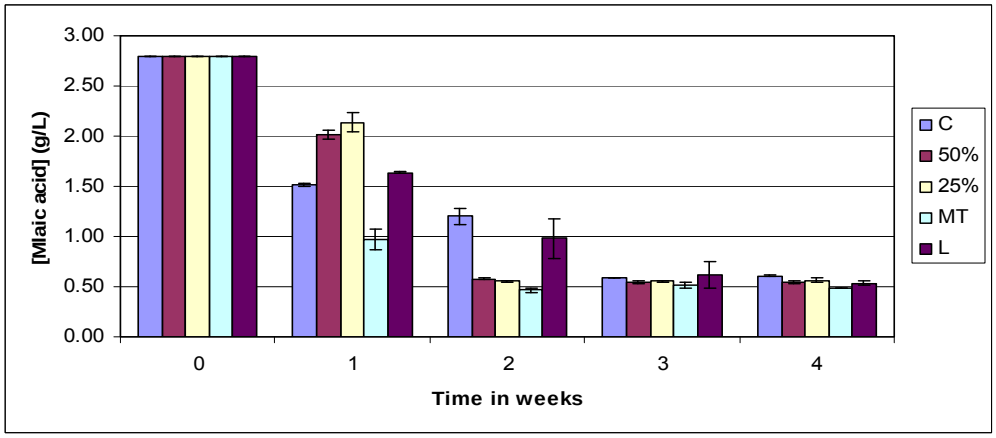


**Figure 3.3** Degradation of malic acid during MLF in Merlot (2005) where different inoculation treatments were evaluated. All treatments were inoculated with Viniflora®CH35. C represents the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represent the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatments that were inoculated from the control samples after MLF have finished. Error bars indicate the standard deviation for three repeats.

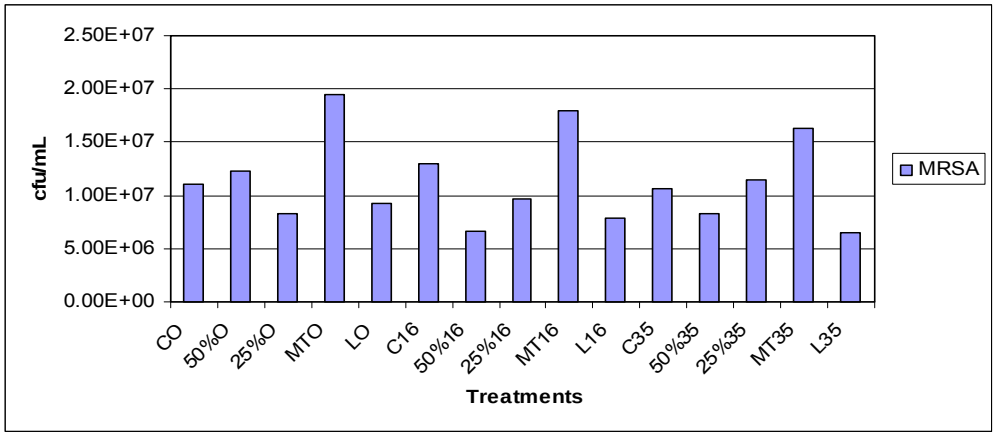


**Figure 3.4** Cell concentrations at the end of MLF for the control and treatments of the Merlot 2005 inoculated with all three starter cultures. O, 16 and 35 represents treatments inoculated with Viniflora®oenos, CH16 and CH35. C are the control samples that were inoculated with 100% of the recommended dosage ($10^6$ cfu/mL); 50% and 25% represents the samples that were inoculated at that percentage of the recommended dosage; MT are the mother tank treatments; L are the treatments that were inoculated with the lees from the control samples after MLF have concluded. Each bar indicates the average of the cell concentrations for the three replicates.

The biogenic amine levels were measured at the end of MLF for the Merlot 2005 (**Table 3.3).** No histamine or cadaverine was detected; except for the L treatments of all three starter cultures that showed low levels of histamine (between 1-2 mg/L) and the L treatment with Viniflora®CH16 that showed levels of cadaverine (0.405 mg/L). These levels of histamine found in the L treatments of the Merlot 2005 were below average for South African red wine, which is approximately 4.8 mg/L (Cilliers and Van Wyk, 1985). It is also below the upper limits for histamine that have been recommended by various countries which are (mg/L): Germany, 5; Holland, 3; Finland, 5; Belgium, 5-6; France, 8; Switzerland and Austria, 10 (Busto *et al*., 1996; Lehtonen, 1996). The L treatment had the longest MLF time in weeks. The L treatments probably had the highest number of naturally occurring LAB and may therefore contain more isolates with amino acid decarboxylase activity. The L treatments showed the highest diversity in the production of the biogenic amines and also the highest amount for two of the biogenic amines. Tyramine was also sporadically detected in the C, 50% and L treatments with Viniflora®oenos, the 50% with Viniflora®CH16 and the MT and L treatments with Viniflora®CH35. These levels ranged from 0.5-1.1 mg/L which was higher than the average level of tyramine in South African red wines (0.5 mg/L) (Cilliers and Van Wyk,

1985). The putrescine levels ranged between 4.3 mg/L and 7.7 mg/L for all the treatments except the L with Viniflora®oenos and Viniflora®CH16, where the level was found to be almost double that. In Swedish red wine, putrescine was found to be present at higher levels than the other BA's (Gafner, 2002).

Putrescine had been found to be mainly associated with the grape or the must (Bertoldi *et al*., 2004; Marcobal *et al*., 2006).

**Table 3.3** Biogenic amines, concentration in mg/L, measured at the end of MLF for all three starter cultures (O Viniflora®oenos; 16 Viniflora®CH16; 35 Viniflora®CH35) and different treatments in Merlot 2005. Each level indicates the average of 2 repeats. C represents the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represent the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatments that were inoculated from the control samples after MLF have finished.

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| | Histamine | Tyramine | Putrescine | Cadaverine |
|---|---|---|---|---|
| CO | nd* | 0.54 | 6.28 | nd |
| C16 | nd | nd | 7.28 | nd |
| C35 | nd | nd | 7.68 | nd |
| 50%O | nd | 0.91 | 4.72 | nd |
| 50%16 | nd | 0.72 | 4.79 | nd |
| 50%35 | nd | nd | 4.84 | nd |
| 25%O | nd | nd | 4.30 | nd |
| 25%16 | nd | nd | 5.48 | nd |
| 25%35 | nd | nd | 4.72 | nd |
| MTO | nd | nd | 6.37 | nd |
| MT16 | nd | nd | 5.97 | nd |
| MT35 | nd | 0.71 | 5.98 | nd |
| LO | 2.02 | 1.12 | 12.4 | nd |
| L16 | 1.94 | nd | 13.4 | 0.41 |
| L35 | 0.97 | 0.59 | 7.18 | nd |

*not detected

The malic acid degradation for the Merlot 2006 showed the same tendency than in 2005 with regards to the MT treatment that performed MLF the fastest, but in this case for all three starter cultures used (**Figures 3.5, 3.6** and **3.7**).

For the samples inoculated with Viniflora®oenos at week 1 the MT treatment showed a malic acid degradation percentagetion of 45% followed by the Lees and CV treatments with a reduction of 17% and 12% respectively. At this stage the other treatments had a reduction rate of between 1% and 9% (**Figure 3.5**). The MT treatment continued to have

the highest reduction during week 2 (83%) and no malic acid was detected for this treatment in week 3. Therefore the MT finished MLF two weeks before all the other treatments.

During week 2 the malic acid degradation percentages for the CV, 50%V and 25%V treatments were 67%, 64% and 13% respectively, which were a much higher reduction level than their counterparts that were not treated with Velcorin before inoculation. C, 50% and 25% had a reduction percentage of only 29%, 16% and 9% respectively. It is possible that in the instances where Velcorin was used prior to inoculation the interaction or competition with the natural occurring LAB was reduced, which enabled the starter cultures to dominate the fermentation and conduct MLF faster. The L treatment had the third highest reduction percentage at this stage with 55% and the S treatment had the lowest reduction rate of 4%.

During week 3 the reduction levels of the different treatments started to become more or less equal. The CV, 50%V, 25%V and L were the treatments with the highest reduction percentage of approximately 80%, followed by the C, 50%, 25% and S treatments with approximately 72%. At week 4 all the treatments had the same reduction level and the end of MLF was reached.

For the Viniflora®CH16 inoculations during the first week of MLF the MT reduced the malic acid concentration with approximately 65%, whilst the other treatments reduced the malic acid with less than 11% (**Figure 3.6**). During week 2 the C, 50% and 25% reflects the difference in the inoculation rate of these treatments. CV and 50%V, the samples that received Velcorin before inoculation with the starter culture, showed a more reduced malic acid concentration (75% and 74% respectively) than its counterparts without Velcorin, C and 50% (68% and 62%). This was a difference of approximately 10%. The 25%V and S had basically not started MLF in week 2 with a malic acid reduction of only 10% and 4% respectively. In weeks 3 and 4 no malic acid was detected in the MT and also in the L at week 4.

For the Viniflora®CH35 inoculations the MT treatment again had the fastest reduction of malic acid over week 1 and 2 (56% and 84%) and no malic acid was detected in week 3 (**Figure 3.7**). The L treatment had the second highest malic acid reduction percentage followed by the treatments that received Velcorin (CV, 50%V and 25%V). The C, 50%, 25% and S treatments had a reduction of between 0 and 5% at this stage. This pattern continued through week 2. In week 3 the CV, 50%V and L showed a reduction of 80%,

followed by the 25%V treatment at 74% and then the C, 50%, 25% and S treatments with a 70% reduction of malic acid. Week 4 marked the end of MLF for all the treatments (except the MT which was already finished with MLF after week 2), and the level of malic acid reduction was the same for all treatments at this stage.

In all three cases (three starter cultures) it is evident that the 25% of the recommended dosage had a longer lag phase before the onset of the MLF.

The cell numbers obtained from the MRS and MRS A media each week since the inoculation of MLF were more or less the same (**Table 3.4**) in the case of all three starter cultures. The cell numbers of the MT treatments correlated with the fast reduction in malic acid concentration since the initial cell numbers (Week 3, **Table 3.4**) for this treatment was approximately $10^3$ cfu/mL more than for the 50% and 25% (Week1, **Table 3.4**) treatments and also slightly higher than the other treatments. From the cell numbers it is also evident that the LAB in the spontaneous MLF and the 25%35 treatments had a similar and longer lag phase than the other treatments. Representative samples of colonies were removed from the two different media and examined microscopically; it was found that the colonies were similar cocci and most likely *O. oeni*.

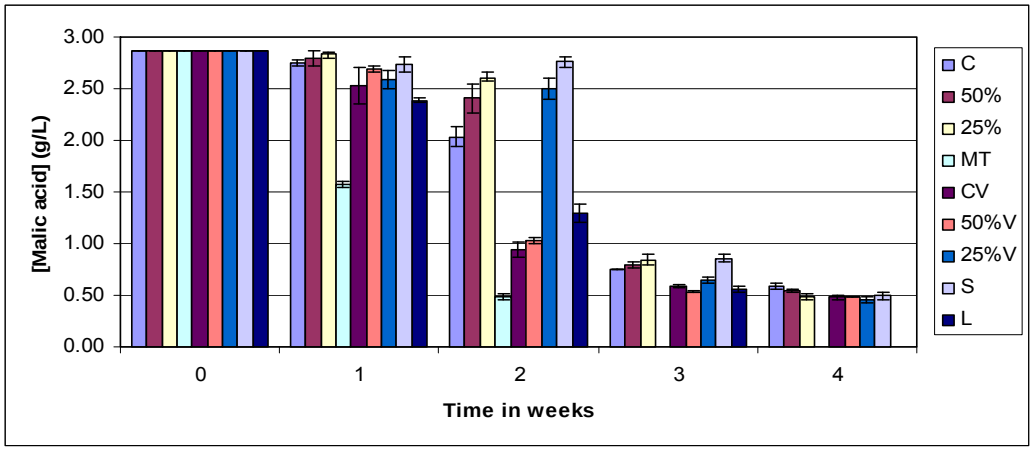


**Figure 3.5** Degradation of malic acid during MLF in Merlot (2006) where different inoculation treatments were evaluated. All treatments were inoculated with Viniflora®oenos except the spontaneous fermentation(S). C represents the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represent the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatments that were inoculated from the control samples after MLF have finished; V represent samples that was treated with Velcorin before MLF inoculation. Error bars indicate the standard deviation for three repeats.

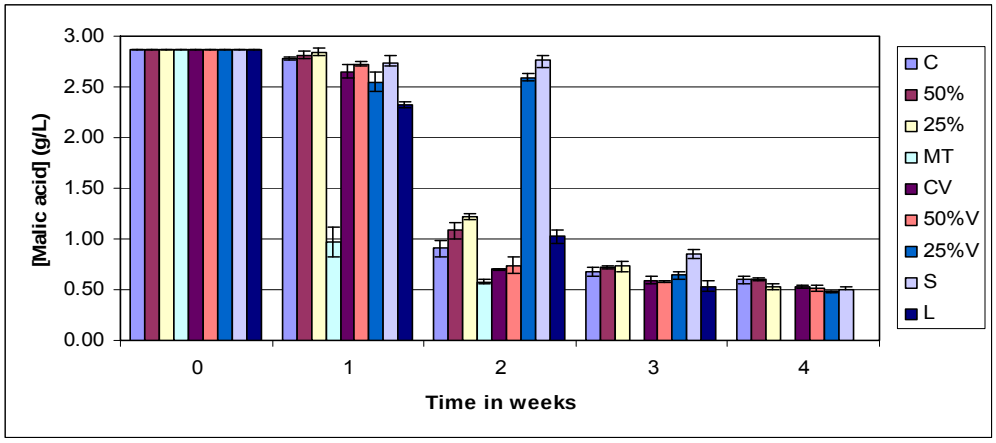


**Figure 3.6** Degradation of malic acid during MLF in Merlot (2006) where different inoculation treatments were evaluated. All treatments were inoculated with Viniflora®CH16 except the spontaneous fermentation (S). C represents the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represent the samples that were inoculated at that percentage of the recommended dosage for CH16; MT represents the mother tank treatments; L represents the lees treatments that were inoculated from the control samples after MLF have finished; V represent samples that was treated with 250 ppm Velcorin before MLF inoculation. Error bars indicate the standard deviation for the three repeats.

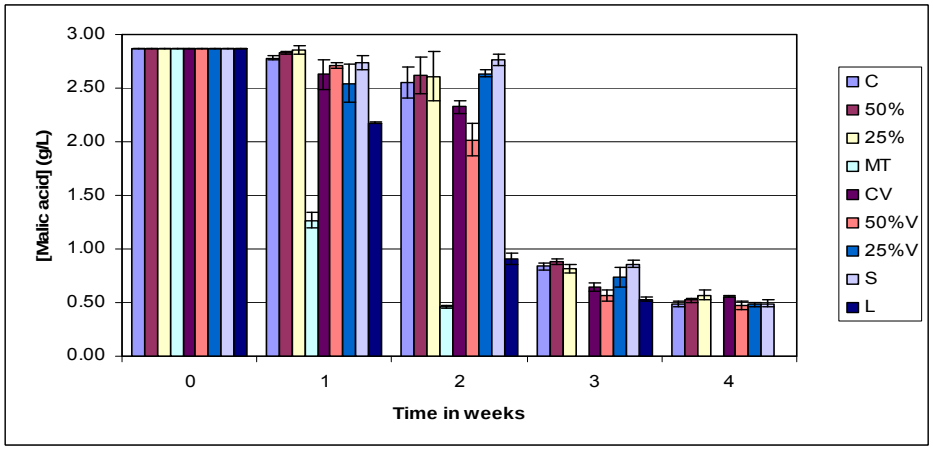


**Figure 3.7** Degradation of malic acid during MLF in Merlot (2006) where different inoculation treatments were evaluated. All treatments were inoculated with Viniflora®CH35 except the spontaneous fermentation (S). C represents the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represent the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatments that were inoculated from the control samples after MLF have finished; V represent samples that was treated with Velcorin before MLF inoculation. Error bars indicate the standard deviation for three repeats.

**Table 3.4** Cell numbers of Merlot 2006 wines obtained weekly since the inoculation of MLF in cfu/mL. MLF was induced with Viniflora®oenos (O), Viniflora®CH16 (16) and Viniflora®CH35 (35), except for the spontaneous MLF (S). C represents the control samples that were inoculated with 100% of the recommended dosage ($10^6$ cfu/mL); 50% and 25% represent the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatments that were inoculated from the control samples after MLF have finished; V represent samples that was treated with Velcorin before MLF inoculation. Each cell number represents the mean of the triplicate.

| | Week 1 | | Week 2 | Week 3 | | Week 4 | |
|---|---|---|---|---|---|---|---|
| | MRS | MRSA | MRSA | MRS | MRSA | MRS | MRSA |
| CO | 2.13E+05 | 2.61E+05 | 3.23E+06 | 1.32E+06 | 4.15E+06 | 2.77E+04 | 2.32E+06 |
| C16 | 5.77E+04 | 6.13E+04 | 6.25E+06 | 8.87E+05 | 3.15E+06 | 1.23E+05 | 1.17E+06 |
| C35 | 1.44E+03 | 4.63E+04 | 2.87E+05 | 1.09E+06 | 5.20E+06 | 8.25E+05 | 6.04E+06 |
| 50%O | 6.03E+04 | 1.17E+05 | 6.55E+05 | 3.62E+06 | 4.49E+06 | 2.25E+06 | 3.67E+06 |
| 50%16 | 1.83E+03 | 2.70E+04 | 4.64E+06 | 1.31E+06 | 1.98E+06 | 4.75E+05 | 2.01E+06 |
| 50%35 | 1.53E+03 | 2.07E+04 | 1.65E+06 | 1.36E+06 | 3.33E+06 | 4.87E+05 | 1.14E+07 |
| 25%O | 1.42E+05 | 4.90E+04 | 4.21E+05 | 2.78E+06 | 2.89E+06 | 9.07E+05 | 5.65E+06 |
| 25%16 | 4.27E+03 | 1.93E+04 | 3.50E+06 | 4.34E+06 | 3.90E+06 | 9.82E+05 | 4.24E+06 |
| 25%35 | 3.47E+03 | 1.40E+04 | 3.23E+04 | 6.45E+05 | 5.07E+06 | 2.29E+05 | 1.08E+07 |
| MTO | 9.99E+05 | 2.87E+06 | 9.87E+04 | 3.48E+06 | | | |
| MT16 | 1.97E+06 | 2.36E+06 | 1.23E+06 | 4.14E+06 | | | |
| MT35 | 1.23E+06 | 4.82E+06 | 2.41E+06 | 9.19E+06 | | | |
| LO | 1.10E+07 | | | | | | |
| L16 | 1.65E+07 | | | | | | |
| L35 | 9.27E+06 | | | | | | |
| CVO | 7.67E+04 | 4.55E+05 | 2.15E+06 | 2.67E+06 | 3.40E+06 | 1.51E+06 | 5.44E+06 |
| CV16 | 2.90E+04 | 3.87E+04 | 3.16E+06 | 6.47E+05 | 1.37E+06 | 1.17E+06 | 2.86E+06 |
| CV35 | 2.10E+03 | 2.93E+04 | 7.27E+05 | 2.83E+06 | 3.40E+06 | 2.49E+05 | 4.68E+06 |
| 50%VO | 1.45E+05 | 1.13E+05 | 5.26E+06 | 1.68E+06 | 4.07E+06 | 8.12E+05 | 6.48E+06 |
| 50%V16 | 2.17E+04 | 1.08E+06 | 4.51E+06 | 6.72E+06 | 2.24E+06 | 1.68E+05 | 7.03E+05 |
| 50%V35 | 1.05E+04 | 1.77E+04 | 1.83E+06 | 3.91E+06 | 4.30E+06 | 0.00E+00 | 6.67E+05 |
| 25%VO | 6.03E+03 | 1.31E+07 | 2.11E+06 | 9.60E+06 | 1.34E+07 | 1.76E+06 | 8.23E+06 |
| 25%V16 | 5.04E+03 | 8.71E+06 | 1.53E+05 | 1.42E+07 | 2.26E+07 | 2.20E+06 | 6.05E+06 |
| 25%V35 | 8.37E+02 | 2.00E+06 | 1.01E+05 | 4.92E+06 | 6.07E+06 | 7.21E+05 | 6.27E+06 |
| S | 8.87E+05 | 3.13E+03 | 3.47E+04 | 3.82E+06 | 8.10E+06 | 1.47E+07 | 5.42E+07 |

### 3.3.3 Pinotage 2005 and 2006

During the 2005 season, the fermentation results of the Pinotage were inconsistent and therefore no conclusions could be made (data not shown).

For the Pinotage 2006 the L treatments for all three starter cultures used, had the fastest reduction of malic acid one week after inoculation (**Figures 3.8, 3.9** and **3.10**). LO, L16 and L35 treatments showed a reduction of 68%, 79% and 75% respectively, whilst the malic acid reduction varied between 0.8% and 11% for the other treatments.

At week two the MT treatments reached the same level than the L treatments for all three starter cultures and showed virtually the same malic acid reduction percentage (81%-84%). Also at week 2 the other treatments for Viniflora®oenos and Viniflora®CH16 showed a higher reduction in malic acid than at week one with C > 50% > 25% treatment, but the reduction were still lower than for the L and MT treatments at this stage (**Figures 3.8** and **3.9**). The C, 50% and 25% treatments with Viniflora®oenos had a higher reduction of malic acid than the Viniflora®CH16 samples in week 2, with a difference of 2.5%, 17% and 20% respectively (**Figures 3.8** and **3.9**). The C35, 50%35 and 25%35 (samples that were inoculated with Viniflora®CH35) only had a malic acid reduction of < 3% at week 2 (**Figure 3.10**).

Week 3 marked the stage where all the treatments that were inoculated with Viniflora®oenos and Viniflora®CH16 reached more or less the same level of malic acid reduction. These degradation percentages for CO/16, 50%O/16 and 25%O/16 were approximately 82% and for the LO/16 and MTO/16 treatments it were approximately 85% (**Figures 3.8** and **3.9**). During week 3 the treatments that were inoculated with Viniflora®CH35 showed the same level of malic acid reduction for the MT and L treatments than the other two starter cultures at this stage (±85%) (**Figure 3.10**). Week 3 also marked the start of MLF (malic acid degradation) for the C, 50% and 25% treatments that were inoculated with Viniflora®CH35, with a reduction in malic acid of 14%, 9% and 5% respectively.

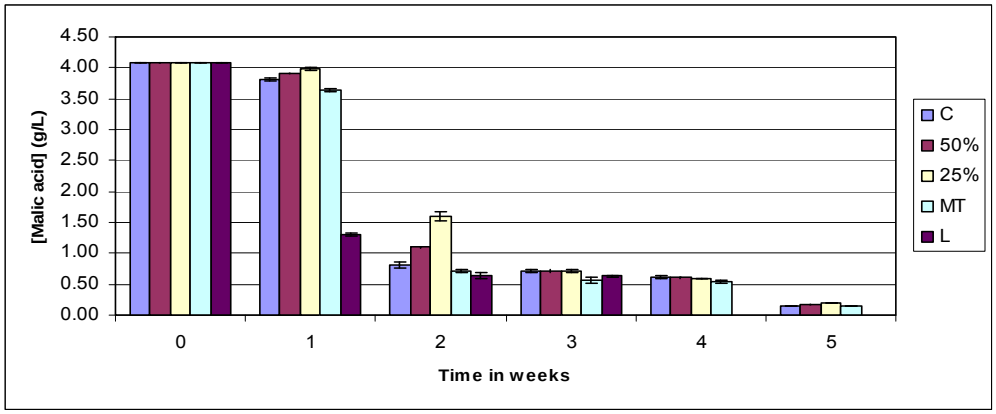


**Figure 3.8** Degradation of malic acid during MLF in Pinotage (2006) where different inoculation treatments were evaluated. All treatments were inoculated with Viniflora oenos. C represents the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represent the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatments that were inoculated from the control samples after MLF have finished. During week 4 and 5 no malic acid was detected for the L treatment. Error bars indicate the standard deviation for the three repeats.

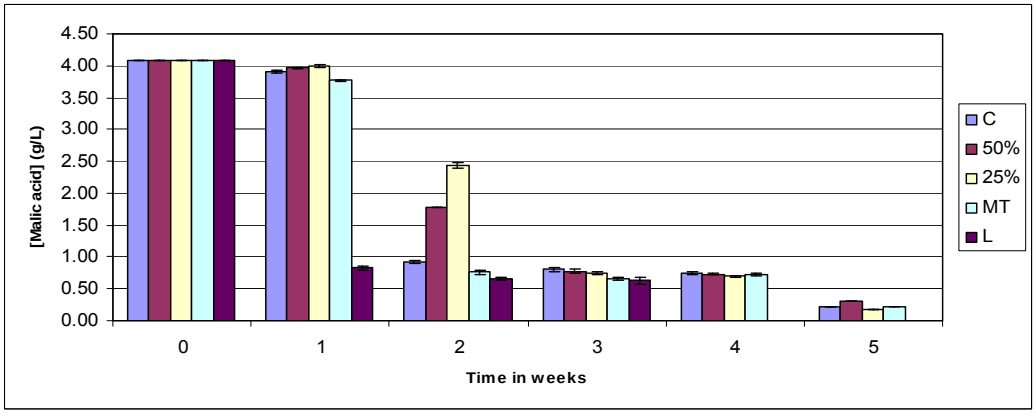


**Figure 3.9** Degradation of malic acid during MLF in Pinotage (2006) where different inoculation treatments were evaluated. All treatments were inoculated with CH16. C are the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represent the samples that were inoculated at that percentage of the recommended dosage for CH16; MT are the mother tank treatments; L are the treatments that were inoculated from the control samples after MLF have concluded. Error bars indicate the standard deviation for the three repeats.

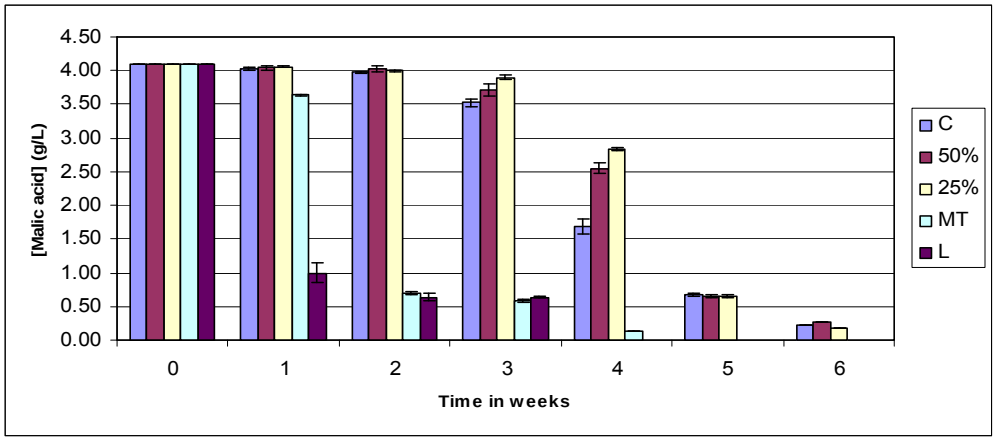


**Figure 3.10** Degradation of malic acid during MLF in Pinotage (2006) where different inoculation treatments were evaluated. All treatments were inoculated with CH35. C are the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represent the samples that were inoculated at that percentage of the recommended dosage for CH35; MT are the mother tank treatments; L are the treatments that were inoculated from the control samples after MLF have concluded. Error bars indicate the standard deviation for the three repeats

At week 4 in the L treatment for all three starter cultures no malic acid was detected and therefore it had the fastest malic acid degradation for all the treatments of Pinotage 2006. The MT, C, 50% and 25% treatments with Viniflora®oenos and Viniflora®CH16 reached approximately the same level of malic acid degradation of 85% and 82% respectively. This tendency of equal levels of malic acid degradation for these treatments continued through week 5 where it reached 96% and 95% marking the end of MLF for these two starter cultures and treatments mentioned (**Figures 3.8** and **3.9**). For the treatments with Viniflora®CH35 at week 4 the malic acid degradation was highest for the MT treatment (±97%), followed by the C (59%), 50% (38%) and 25% (31%) treatments (**Figure 3.10**). In week 5 there was no malic acid detected for the MT35 treatment and the C35, 50%35 and 25%35 treatments reached approximately the same level of malic acid degradation of 84%. This tendency carried over to week 6 where the degradation level was approximately 95%, which marked the end of MLF for these treatments.

The cell numbers that were obtained at the end of MLF for all three starter cultures and all the treatments are shown in **Figure 3.11**. Cell numbers were obtained from MRS A media in all instances and were always higher than the cell numbers obtained from MRS media. This indicates strongly that *O. oeni* played the major role in performing MLF or malic acid degradation during this experiment. The C, 50% and 25% treatments with Viniflora®CH35 displayed a long lag phase before the onset of malic acid degradation

(approximately 2 weeks longer than for the same treatments with the other two starter cultures). ViniﬂoraCH35 starter culture was developed by the manufacturers for use in white wines and therefore this lag phase could be due to the more complex matrix of red wines. The cell numbers obtained from the C35, 50%35 and 25%35 treatments indicated the possible presence of high levels of other LAB species as was also found in the CO, MTO, C16 and 50%16 treatments. In the Pinotage 2006 the dosage effect is clearer with regard to cell numbers and the rate of MLF in the first weeks than in the Merlot. The question that needs to be answered in future is the dominance of the inoculated starter cultures in the 50 and 25% inoculation. The L treatments showed the lowest level of cell numbers at the end of MLF which could be due to a faster cell death cycle that could have occurred in this treatment, since the starter cultures were already at optimum cell capacity when it was inoculated into the wines for the L treatments.

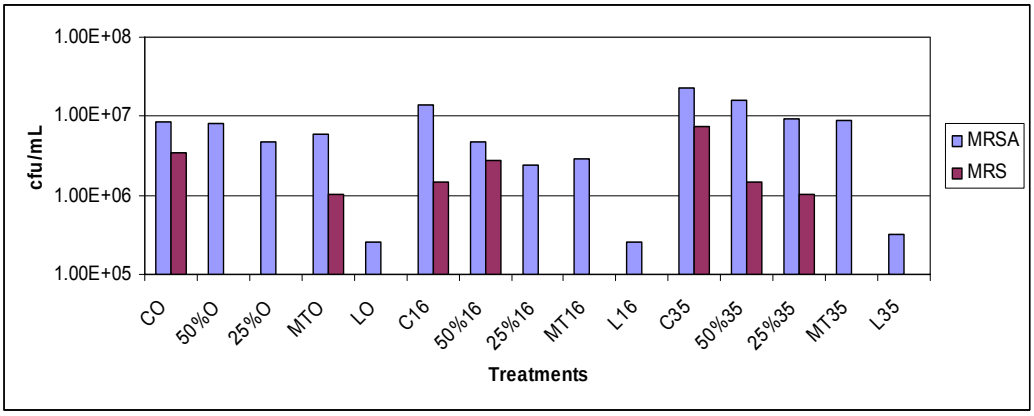


**Figure 3.11** Cell numbers at the end of MLF for the control and treatments of the Pinotage 2006. This figure represents all three starter cultures Viniflora®oenos (O), Viniflora®CH16 (16) and Viniflora®CH35 (35). C represents the control samples that were inoculated with 100% of the recommended dosage ($10^6$ cfu/mL); 50% and 25% represents the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatments that were inoculated from the control samples after MLF had finished. Each bar indicates the average of the cell concentrations for the three replicates.

### 3.3.4 Cabernet Sauvignon 2005 and 2006

During the 2005 season, the fermentation results of the Cabernet Sauvignon were inconsistent and therefore no conclusions could be made (data not shown), but the biogenic amines measurements at the end of MLF will be shown.

For the Cabernet Sauvignon 2006 wine showed that the MT and L treatments, for all three starter cultures, finished malic acid degradation the fastest (**Figures 3.12, 3.13** and **3.14**).

After the first two weeks of MLF, the treatments that were inoculated with Viniflora®oenos (**Figure 3.12**) showed a reduction in malic acid in the following order: C>50%>25%>L>MT. At week 3 this order changed only with regards to the 25% treatment, which had the lowest reduction of malic acid at this stage. At week 4 the scenario was changed completely since here the L, MT and C treatments were virtually the same (85%, 84%, 83%), whilst the 50% had a reduction of 77% and the 25% again had the lowest reduction of malic acid at only 54%. At week 5 no malic acid were detected in the MT and L treatments whilst the C, 50% and 25% were more or less at the same level of malic acid degradation (±84%).

The treatments that were inoculated with Viniflora®CH16 (**Figure 3.13**) showed the following order from highest to lowest with regards to the percentage of malic acid reduction over the first two weeks of MLF: C>L>50%>25%>MT. At week 3 this order of malic acid reduction rate changed to C and MT being at the same level (±74%), followed by L at 73% with the 50% and 25% treatments being at 53% and 33% respectively. At week 4 the MT and L treatment had the highest percentage reduction of malic acid (85%) with C at 80% and the 50% and 25% treatment both at 71%. Week 5 marked the end of MLF for the MT and L treatments, since no malic acid was detected for these treatments, whilst the C, 50% and 25% reached approximately the same level of malic acid reduction at 85%.

For the treatments that were inoculated with Viniflora®CH35 (**Figure 3.14**) the reduction of malic acid occurred with basically the same trends than for the Viniflora®CH16 inoculations. There were basically two differences between these two starter cultures. Firstly in week 2 the order were C and L at the same level of malic acid reduction, followed by 50% and then 25% and MT at the same level with the lowest reduction (C>L>50%>MT>25% for Viniflora®CH16). The second difference were that the level of malic acid reduction were lower over the first three weeks for the Viniflora®CH35

inoculations, even though the patterns of malic acid reduction were similar. The same reasons for the lag phase with Viniflora®CH35 applies here that was mentioned in the case of the Merlot 2006 wine. Strain differences with regards to the conducting of MLF have been previously reported and are a common occurrence. MLF starter cultures can react different when induced into the same wines as a result of various reasons, which include winemaking techniques used as well as physical and chemical parameters of wine. In 1977 and 1980 Beelman *et al*. found that that the *O. oeni* strain PSU-1 was more effective than ML-34 (also *O. oeni*) in Pennsylvannia red wines. The PSU-1 strain was isolated from Pennsylvannia grapes (Eastern US), whilst the ML-34 strain was isolated from the warmer southern region of California. The reason for this was speculated to be that PSU-1 had a better adaptation in wines with higher acidity (lower pH) due to higher malic acid levels like those in Pennsylvannia red wines when compared to wines from the warmer Californian grape regions.

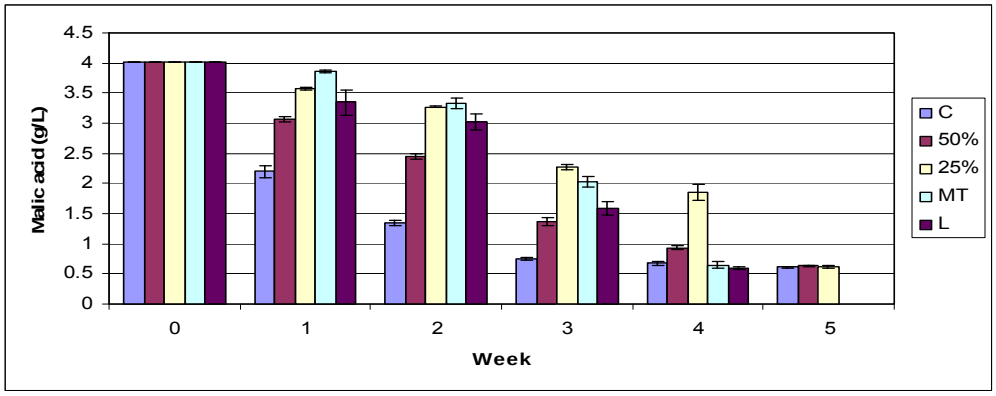


**Figure 3.12** Degradation of malic acid during MLF in Cabernet Sauvignon (2006) where different inoculation treatments were evaluated. All treatments were inoculated with Viniflora®oenos. C represents the control samples that were inoculated with 100% of the recommended dosage ($10^6$ cfu/mL); 50% and 25% represents the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatment that were inoculated from the control samples after MLF had finished. At week 5 no malic acid were detected for the MT and L treatments. Error bars indicate the standard deviation for the three repeats.

At the end of MLF cell numbers were only obtained from MRS A media, although the samples were also enumerated on MRS media (**Figure 3.15**). This indicates that *O. oeni* was most likely the major LAB involved in MLF for this cultivar. At the end of MLF all the treatments showed cell number > $10^6$ cfu/mL, except for the CO treatment than had a slightly lower cell number. No correlations could be drawn between the faster rate of malic

acid reduction of the MT and L treatments and the cell numbers at this stage as cell counts were only done at the end of MLF.

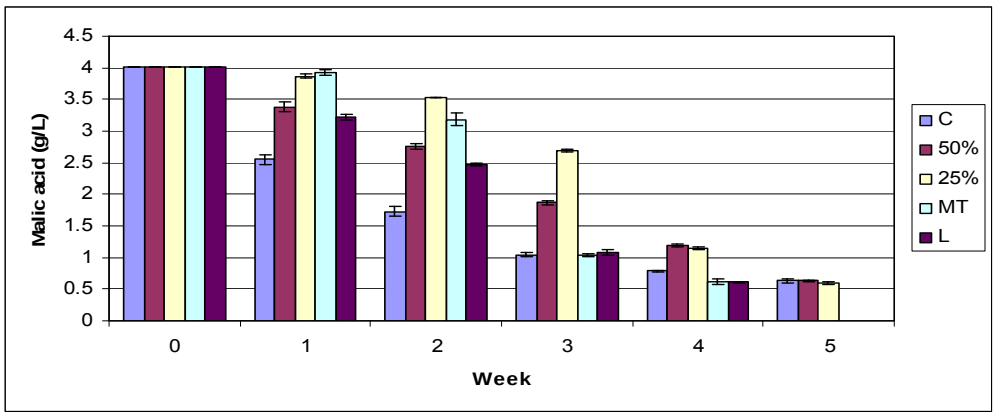


**Figure 3.13** Degradation of malic acid during MLF in Cabernet Sauvignon (2006) where different inoculation treatments were evaluated. All treatments were inoculated with Viniflora®CH16. C represents the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represents the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatment that were inoculated from the control samples after MLF had finished. At week 5 no malic acid were detected for the MT and L treatments. Error bars indicate the standard deviation for the three repeats.

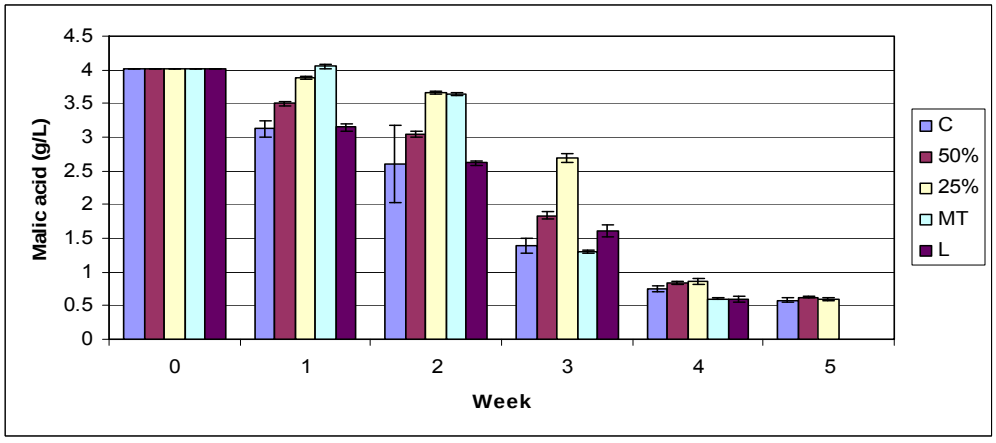


**Figure 3.14** Degradation of malic acid during MLF in Cabernet Sauvignon (2006) where different inoculation treatments were evaluated. All treatments were inoculated with Viniflora®CH35. C represents the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represents the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatment that were inoculated from the control samples after MLF have finished. At week 5 no malic acid were detected for the MT and L treatments. Error bars indicate the standard deviation for the three repeats.

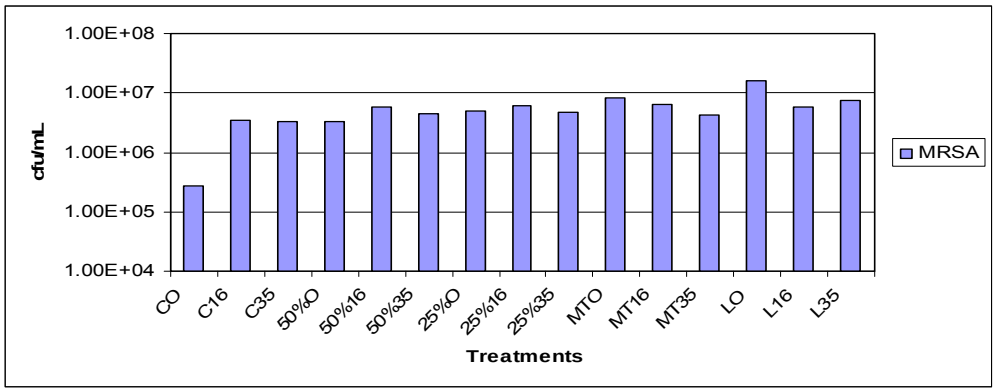


**Figure 3.15** Cell concentrations at the end of MLF for the control and treatments of the Cabernet Sauvignon 2006. This figure represents all three starter cultures Viniflora®oenos (O), Viniflora®CH16 (16) and Viniflora®CH35 (35). C represents the control samples that were inoculated with 100% of the recommended dosage; 50% and 25% represents the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatments that were inoculated from the control samples after MLF have finished. Each bar indicates the average of the cell concentrations for the three replicates.

The biogenic amine levels that were measured at the end of MLF for the Cabernet Sauvignon 2006 showed no large differences, except for the lees treatments regarding histamine (**Table 3.5**). No tyramine was produced in any of the treatments during MLF. The histamine levels range between 2 and 5 mg/L and was formed in all treatments except the lees treatments. As mentioned earlier the average level of histamine in South African red wines were found to be approximately 4.8 mg/L, therefore these levels obtained are within this range. The putrescine levels ranged between 29 and 42 mg/L. Cadaverine was produced in all the treatments and the levels varied between 0.8 and 1.3 mg/L. BA levels, especially the levels of histamine, in wine have come to play an important role regarding importing and exporting of wines.

**Table 3.5** Biogenic amines, concentration in mg/L, measured at the end of MLF for all three starter cultures (O Viniflora®oenos; 16 Viniflora®CH16; 35 Viniflora®CH35) and different treatments in Cabernet Sauvignon 2005. Each level indicates the average of 2 repeats. C represents the control samples that were inoculated with 100% of the recommended dosage (> 5 x $10^6$ cfu/mL); 50% and 25% represent the samples that were inoculated at that percentage of the recommended dosage; MT represents the mother tank treatments; L represents the lees treatments that were inoculated from the control samples after MLF have finished.

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| | Histamine | Tyramine | Putrescine | Cadaverine |
|---|---|---|---|---|
| CO | 5.029 | nd | 35.332 | 1.307 |
| C16 | 3.491 | nd | 36.366 | 1.285 |
| C35 | 4.254 | nd | 36.691 | 1.297 |
| 50%O | 4.537 | nd | 34.509 | 1.235 |
| 50%16 | 3.590 | nd | 37.847 | 1.244 |
| 50%35 | 3.630 | nd | 36.300 | 1.143 |
| 25%O | 3.406 | nd | 29.950 | 1.020 |
| 25%16 | 3.877 | nd | 34.587 | 1.140 |
| 25%35 | 3.801 | nd | 37.986 | 1.213 |
| MTO | 2.626 | nd | 33.780 | 1.200 |
| MT16 | 2.269 | nd | 33.550 | 1.169 |
| MT35 | 2.083 | nd | 31.781 | 0.843 |
| LO | nd* | nd | 34.124 | 1.036 |
| L16 | nd | nd | 34.768 | 1.012 |
| L35 | nd | nd | 42.476 | 1.009 |

* not detected

## 3.4 CONCLUSION

The stretching of MLF starter cultures is used by winemakers in practice to lower costs with regards to MLF. During this study the stretching treatments all resulted in a complete MLF. Differences between the rates at which these treatments performed ~~degraded~~ malic acid degradation were observed, especially in the initial stages of MLF (Weeks 1 and 2). In most cases the MT and L treatments performed malic acid degradation the fastest. In the Merlot 2005 the MT treatment inoculated with Viniflora®oenos finished MLF one week before the other treatments. For the Merlot 2006 the MT and L treatments finished MLF after two and three weeks respectively, before the other treatments for all three starter cultures used in the study. In Cabernet Sauvignon 2006 the MT and L treatments for all three starter cultures was finished a week before the other treatments. In the Pinotage the L treatments with all three starter cultures was finished two weeks before the other

treatments whilest the MT treatment with Viniflora®CH35 was finished one week before the other treatments. The increased degradation in malic acid for these two2 treatments could be explained due to the fact that in these treatments the starter culture was pre-exposed to the wine conditions before it was inoculated into the wine. The malolactic activity of the treatments was already at a high level at inoculation, which would explain this higher reduction levels especially after the first week of inoculation. Other results observed for Merlot 2006 were the faster degradation rate for the CV and 50%V treatments when compared to their counterparts that did not receive Velcorin (to sterilise wine) before MLF. The Velcorin could have reduced the natural occurring LAB, which could have enhanced the performance of the commercial starter cultures.

This is interesting good results from the perspective of the winemaker or producer, because any of these methods will result in a successful MLF. This was true Eespecially in the case of the MT treatment that could perform MLF within 3 weeks under these specific conditions. A fast MLF can increase the This could increase the production flow within the cellar to a great extent.

However, wBut when looking more holistically at these results, other aspects which areare extremely important to keep in mind. Firstly, the bacteria used to perform MLF in this study were all from the same supplier and therefore the question arises whether the same results would be found when other starter cultures are used. Secondly, the bacteria that performed the MLF were not identified to species level after MLF, but rather identified on a microscopic level on morphology. Therefore uncertainties do exist with regards to which organisms did indeed perform the MLF and the cell number data were rendered inconclusive. Thirdly, BA's were also present at the end of MLF, while the selected starter cultures are not supposesupposed to do not produce BA's according to the suppliersproducers. This suggests that the natural LAB could have contributed to the process of MLF.

Therefore future work that could extend this study would be the identification of the microorganisms at the end of MLF by using PCR or to evaluate the wines for difference in aroma and volatile compounds that developed during MLF to determine the over-all quality difference of the wines. The layout of the experiment should include a spontaneous MLF in all cases and the cell numbers of the bacteria must be obtained on a weekly basis to follow the evolution of the starter cultures as well as the natural occurring LAB throughout MLF.

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# 4. RESEARCH RESULTS

# Effect of a wine isolated *Lactobacillus* spp. and an *Acetobacter pasteurianus* in combination with a malolactic fermentation starter culture on MLF at different wine pH's

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## ABSTRACT

Must and wine host an array of natural occurring yeasts and bacteria apart from those that are inoculated into the must and wine to promote the processes of alcoholic fermentation and malolactic fermentation. These indigenous microorganisms are able to contribute to the outcome of the wine and over-all wine quality. For the purpose of this study Cabernet Sauvignon wine of the Western Cape region of South Africa was adjusted to pH 3.0, 3.4 and 4.0 after completion of alcoholic fermentation. The wine was inoculated with a combination of a malolactic fermentation starter culture Viniflora®CH16 (CH16) (*Oenococcus oeni*) with a Lactic acid bacterium (isolate 16.7) (*Lactobacillus* spp.) (AB) and an acetic acid bacterium (F14) (*Acetobacter pasteurianus*) (AC) isolated from wine respectively. The controls for the experiment were CH16 (A), isolate 16.7 (B) and the F14 (C) inoculated separately. The rate of inoculation was $10^6$ cfu/mL for the CH16 and $10^4$ cfu/mL for the isolate 16.7 and F14. The progress of MLF and the possible interaction between the starter culture and the other inoculated bacteria was monitored by enumeration on selective media as well as measuring the malic acid degradation weekly using an enzymatic kit. The wine was monitored over a period of 32 days. On day 39 the wines was analysed using FT-IR spectroscopy and the biogenic amine levels was also measured at this stage. The results showed no degradation of malic acid or any viable bacterial cells over the period of 32 days for pH 3 wine. The pH 3.4 had viable bacterial cells over the course of the 32 days but it never reached significant levels to initiate the

degradation of malic acid. In the pH 4.0 wines it was observed that malic acid degradation was the fastest for the combination of CH16 and isolate 16.7, followed by the CH16 control and lastly the combination of CH16 and F14. The isolate 16.7 control did not degrade malic acid. The malic acid concentration as measured by FT-IR spectroscopy confirmed these trends. No significant differences were observed between the levels of biogenic amines between the treatments.

## 4.1 INTRODUCTION

Grape must and wine host a complex microflora comprising of yeasts and various bacteria. The natural occurring microorganisms of wine originate from the grapes and survive on cellar equipment. These organisms are present throughout the winemaking process and must be controlled to ensure wine quality. Alcoholic fermentation (AF) as well as malolactic fermentation (MLF) is processes that are completed by yeasts and lactic acid bacteria (LAB) respectively.

MLF usually occurs after AF, but can sometimes occur earlier. MLF refers to the deacidification of wine by the decarboxylation reaction during which L-malic acid is converted to L-lactic acid (Davis *et al*., 1985). LAB strains isolated from wine belong to the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Wibowo *et al*., 1985). *O*. *oeni* starter cultures commercially prepared to use in the freeze dried form for the induction of malolactic fermentation. Inoculation with such a starter culture, reduces the potential of spoilage by other LAB and/or bacteriophages, ensures a rapid onset of MLF and provide better control over the production of aromatic compounds and therefore wine flavour (Henick-Kling, 1988). Wine pH is one of the most critical factors that influences the growth and viability of bacteria, with an increase in pH the wine environment becomes more stimulatory for growth of certain bacteria. Wines from South Africa, California and Australia, for instance, are from warmer viticultural regions with consequently lower acidity (Davis *et al*., 1985; Kunkee, 1967; Wibowo *et al*., 1985) and therefore a higher pH.

The evolution of natural LAB and acetic acid bacteria (AAB) in wine, have been previously studied and is basically the result of viticultural practices, wine conditions and winemaking techniques. LAB is present on the grape and grape leaves at low concentrations ($10^2$ cells/g) (Lafon-Lafourcade *et al*., 1983; Wibowo *et al*., 1985). After crushing of the grapes this concentration increases to approximately $10^4$ cfu/mL in the juice. *Leuc. mesenteroides*, *O. oeni*, *L. plantarum*, *L. casei* and *P. damnosus* are the

major LAB species present after crushing. These natural occurring LAB and wild yeasts then decrease in cell numbers due to the addition of $SO_2$ (30-50 ppm) after crushing, as well as the inhibiting effect of alcohol that is formed during AF. At the end of AF, LAB starts to grow again and can reach cell concentrations of approximately $10^7$ cfu/mL. At this stage *O. oeni* will dominate in low pH wines (< 3.5) with other LAB such as *P. damnosus,* growing in wines with higher pH, which could result in spoilage of the wine (Davis *et al*., 1985; Wibowo *et al*., 1985).

In a doctoral study by Joyeux (Joyeux, 1983) that was performed on various grape cultivars in three successive years (1978, 1979 and 1980) the following knowledge was obtained with regards to acetic acid bacteria (AAB). AAB are present on grapes, and fluctuate in cell concentration due to the degree at which the grapes are infected. The cell concentration is normally around $10^2$ cfu/mL for healthy grapes with *Gluconobacter oxydans* being the main representative of AAB on such grapes (white and red). *Acetobacter aceti* and to a lesser extent, *A*. *pasteurianus,* become more prevalent as the grapes become spoilt, especially on grapes infected with *Botrytis cinerea*. Freshly pressed must contain about $10^4$ cfu/mL *G*. *oxydans*, which decrease during AF. From this point through the winemaking the levels fluctuate with addition of sulphur dioxide and contact with air, which decrease and increase the levels respectively. AAB produce acetic acid and acetaldehyde in wine. A higher wine pH also leads to higher cell concentrations of AAB and therefore the resulting spoilage components (Du Toit and Lambrechts, 2002; Du Toit and Pretorius, 2002; Joyeux *et al*., 1984).

The aim of this study was to start preliminary work with regards to the impact of a LAB and a AAB on the growth and metabolism of a MLF starter culture (*O. oeni*) and MLF rate at different pH's.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Preparation of samples

Cabernet Sauvignon grapes from the Western Cape wine region was used for this experiment. Approximately 120 kg of these grapes were de-stemmed and 30 ppm $SO_2$ was added. The must were fermented on the skins with 30 g/hL WE 372 yeast (Anchor Yeast Biotechnologies, South Africa). After AF, the skins were pressed and racked to 20 L stainless steel canisters for storage at 4˚C. Before the start of the experiment the wine was brought to room temperature and subsequently sterilised by using 250 ppm Velcorin®

(Bayer, Germany). After sterilization the wine pH was adjusted with tartaric acid or 1N NaOH to the respective pH's of 3.0, 3.4 and 4.0. The wine was then aliquoted into 100 mL sample bottles and closed with a rubber stopper and S-shaped airlocks. The experimental design is showed in **figure 4.1**.

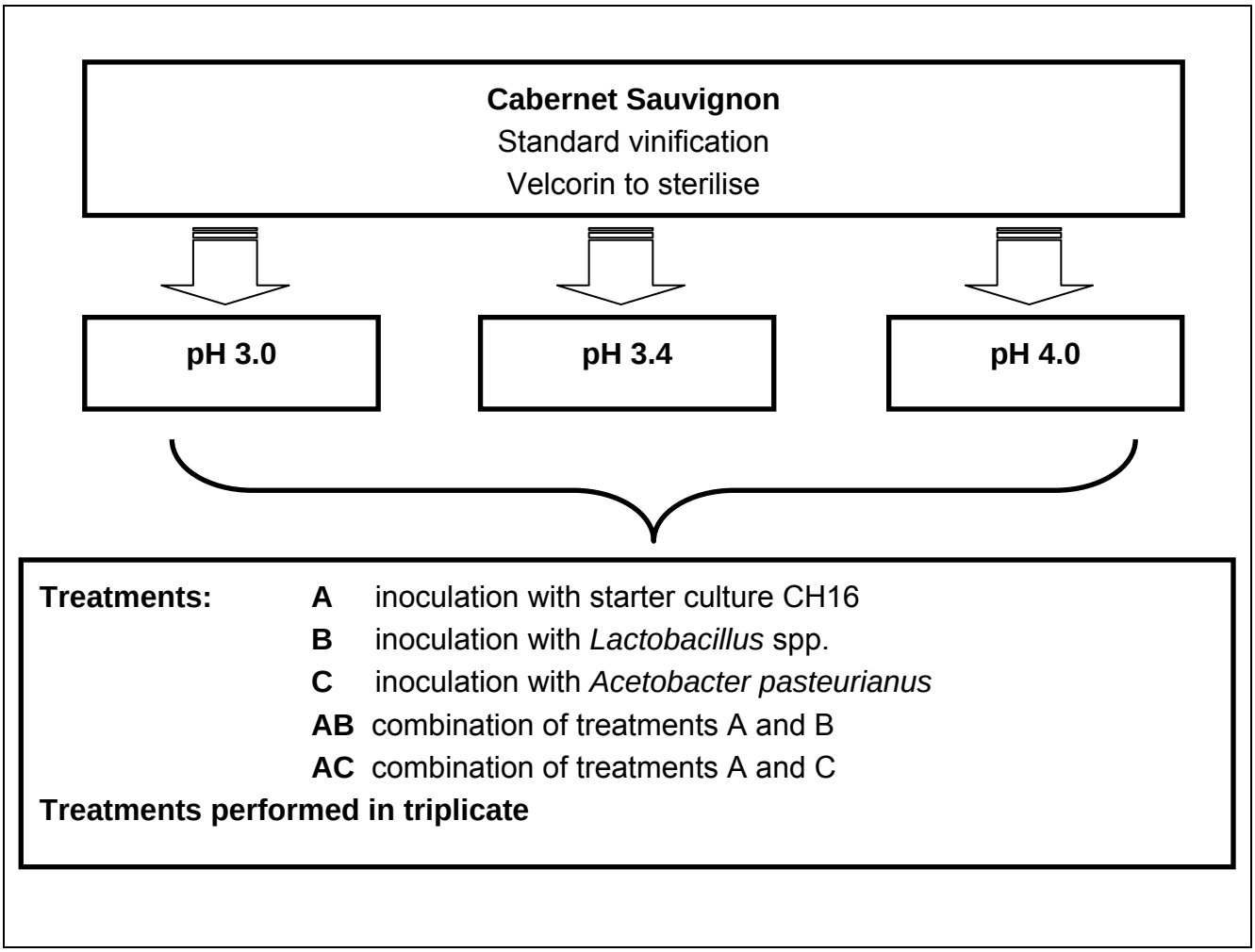


**Figure 4.1** Experimental design.

### 4.2.2 Bacterial strains and culture conditions

Three different bacteria were used during this study namely: Viniflora®CH16, Isolate 16.7 and F14.

Viniflora®CH16 is a MLF starter culture (CH16) (ChrHansen, Denmark) that had been isolated from a Petit Shiraz wine in the Russian River area of California USA. It is a pure culture *O. oeni* in the freeze dried form. CH16 was inoculated at 1.5 g/250 L in the 100 mL wine samples. For enumeration of this culture MRS agar (Biolab, Merck, South Africa) enriched with 20% apple juice (containing no preservatives) (MRSA) (pH5.2) was used. ).

The addition of apple juice and lowering of the pH stimulates the growth of *O. oeni*. Incubation took place under facultative anaerobic conditions at 30˚C (Anaerogen, Oxoid) in an anaerobic jar (Davies Diagnostics (PTY) Ltd).

Isolate 16.7 (16.7) is a *Lactobacillus* spp. from South African wine (Paarl region) (collection of IWBT, Stellenbosch University) was also used as a treatment. This LAB was streaked out from a freeze culture onto pour plates of De Man, Rogosa and Sharpe (MRS) agar (De Man *et al*., 1960). After 5-10 days of incubation under facultative anaerobic conditions (as described for CH16), single colonies from this media was inoculated into 9 mL of liquid MRS. This was allowed to grow for 48h after which the suspension was plated out onto the same media to calculate the cell concentration after 48 hours. The cell concentration was found to be approximately 7.3 x $10^8$ cfu/mL, which was used to inoculate at a rate of $10^4$ cfu/mL into the 100 mL wine samples.

F14 is an *Acetobacter pasterianus* that was isolated from a rebate wine (collection of IWBT, Stellenbosch University) of South Africa. A freeze culture of this strain was streaked out on MRS medium to which 2% v/v ethanol (MRSEtOH) was added after autoclaving. F14 was also inoculated into the wine at a concentration of $10^4$ cfu/mL. For enumeration of F14 MRSEtOH medium as described above was used. Incubation of F14 was done aerobically at 30˚C.

### 4.2.3 Analyses

Enumeration of the wine samples started on day 8 after inoculation and was done every third day up to day 32. Different dilutions of the samples were plated out for the different pH wines. Ten fold dilutions were made by using test tubes filled with 9 mL of distilled, sterile water. The same media mentioned under 4.2.2 was used for the plating. L-Malic was analysed using FT-IR spectroscopy (Foss-Grapescan) before the onset of MLF and at day 39 (end of experiment). These samples were filtered with a Filtration Unit (type 79500, FOSS Electric, Denmark) connected to a vacuum pump. The filter unit uses filter paper circles graded at 20 – 25 µm with diameter 185 mm (Schleicher & Schnell, reference number 10312714). The filtered must were used for FT-IR spectral measurements. A Winescan FT120 equipped with a purpose built Michelson interferometer was used to generate the FT-IR spectra (FOSS Electric A/S, Hillerød, Denmark). Instrument settings included: cell path length of 37 µm, sample temperature set to 40°C, and sample volume of 7 – 8 ml. The sample is pumped through the heat exchanger and the $CaF_2$-lined cuvette. Samples are scanned from 5011 to 926 $cm^{-1}$ at 4 $cm^{-1}$ interval. On day 39

samples were also collected for biogenic amine analysis with high performance liquid chromatography (HPLC) (Distell, South Africa) (Alberto *et al*., 2002).

## 4.3 RESULTS AND DISCUSSION

### 4.3.1 pH 3.0

In the case of pH 3.0 wine no culturable cells of the microorganisms tested were observed after enumeration of the samples through the course of the 32 days sampling period and also no malic acid reduction occured. In 1986 Davis *et al*. already stated that pH has a profound and selective effect on LAB species that grow in wine. A study by Davis *et al*. (1988), to determine the growth rate of LAB at different pH's (3.0 - 5.5) showed that even though some of the *O. oeni* strains that was used did grow at a pH of 3.0, a substantial reduction occurred in the proportion of strains that were able to grow at this pH. Britz and Tracey (1990) also found that a decrease in pH had a negative effect on the growth of *O. oeni*. Liu *et al*. (1995) found that *O. oeni* could grow well at an initial pH of 3.2, but *Lactobacillus plantarum* was much more sensitive and could be inhibited by an initial pH of even 3.5. All these studies were done in media and not wine. AAB are also thought to be inhibited by lower pH values, although the anaerobic conditions probably played a large role here (Du Toit and Pretorius, 2002). Anaerobic conditions as kept during this experiment, even though acetic acid bacteria was also tested, was done because it simulated most likely conditions in the industry.

### 4.3.2 pH 3.4

The pH 3.4 wines did have culturable cells in all the treatments with LAB, but the concentration of the cell numbers was < $10^4$ cfu/mL which is not high enough to initiate the degradation of malic acid (**Table 4.1**). Lonvaud-Funel (1995) stated that malic acid transformation actually begins when the bacterial population is more than about $10^6$ cells/mL. A (CH16 alone), AB (CH16 + isolate 16.7) and AC (CH16 + F14) were the treatments that contained the commercial MLF starter culture. CH16 in the combination treatments (AB and AC) dominated the wine matrix over the first 26 days at least, but all three treatments (A, AB and AC) showed a steady decline in cell numbers over the course of the 32 days. Davis *et al*. (1986, 1988) illustrated that *O. oeni* had a greater tolerance to low pH values and that explains the almost exclusive isolation of this species from wine

with a pH below 3.5. F14 never had any culturable cells for the duration of the 32 day period (Data of not shown in **Table 4.1**). AAB are aerobic and the sample bottles were filled to capacity, therefore the growth of these bacteria was not only restricted by the low pH value, but also by the anaerobic conditions of the samples. The cells of isolate 16.7 in treatment B decreased within the first 8 days after inoculation and was only present again from day 29 onwards and AB (the combination of CH16 and isolate 16.7) decreased to below $10^2$ cfu/mL since inoculation and were only present again from day 29.

**Table 4.1** Cell concentrations obtained from the Cabernet Sauvignon pH 3.4 wine during the course of 32 days for the different treatments. A represent the samples inoculated with CH16; B is the sample set inoculated with isolate 16.7; AB represents the combination treatment of CH16 with isolate 16.7; AC represents the treatment for the combination of CH16 with F14. Each cell represents the average of the triplicates.

| | A | B | AB | AB | AC |
|---|---|---|---|---|---|
| Day | MRSA | MRS | MRSA | MRS | MRSA |
| 8 | 2.37E+03 | nd* | 3.13E+03 | 1.96E+03 | 4.17E+03 |
| 11 | 8.67E+02 | nd | 5.53E+03 | nd | 2.30E+03 |
| 14 | 4.67E+02 | nd | 7.50E+02 | nd | 1.25E+03 |
| 17 | 3.20E+02 | nd | 4.30E+02 | nd | 6.23E+02 |
| 20 | 6.33E+02 | nd | 4.00E+02 | nd | 8.67E+02 |
| 23 | 1.87E+02 | nd | 1.00E+00 | nd | 5.20E+02 |
| 26 | 1.47E+02 | nd | 3.23E+02 | nd | 1.10E+02 |
| 29 | 5.33E+01 | 5.16E+03 | 1.07E+02 | nd | 2.20E+02 |
| 32 | 8.00E+01 | 5.00E+03 | 8.67E+01 | nd | 1.43E+02 |

*not detected

### 4.3.3 pH 4.0

At pH 4.0 malic acid degradation did take place in all the treatments that were inoculated with CH16 (**Figure 4.2**) (A, AB, AC). On day 0 the malic acid level was 3.56 g/L as measured with the Foss Grape Scan. At the end of MLF treatment AB (CH16+isolate 16.7) had the highest malic acid reduction level of 71%, followed by the treatment AC (CH16+F14) and A (CH16) with 64% and 57% malic acid reduction percentage respectively. Treatments B (isolate 16.7) and C (F14) did not show any reduction of malic acid over the period of 39 days. A hypothesis that could be made from this is that in the case of the combination treatments it was in fact the starter culture (CH16) that performed the malic acid degradation. From the malic acid reduction percentages it is clear that the combination treatment AB (CH16 + isolate 16.7), had a higher reduction level than

treatment A (CH16) where the starter culture was inoculated on its own into the wine. This indicates that a possible competition could exist between the organisms when inoculated together, which could force the starter culture to perform better in the medium in order to survive.

These malic acid reduction levels at the end of MLF could also be correlated with the cell numbers obtained from enumeration (**Figure 4.3**), which showed the cell numbers of treatments A, AB and AC to be higher than treatments B and C. In treatments B and C the cell numbers stayed below $10^4$ cfu/mL, and as previously mentioned malic acid degradation only occur when cell number are approximately at $10^6$ cfu/mL.

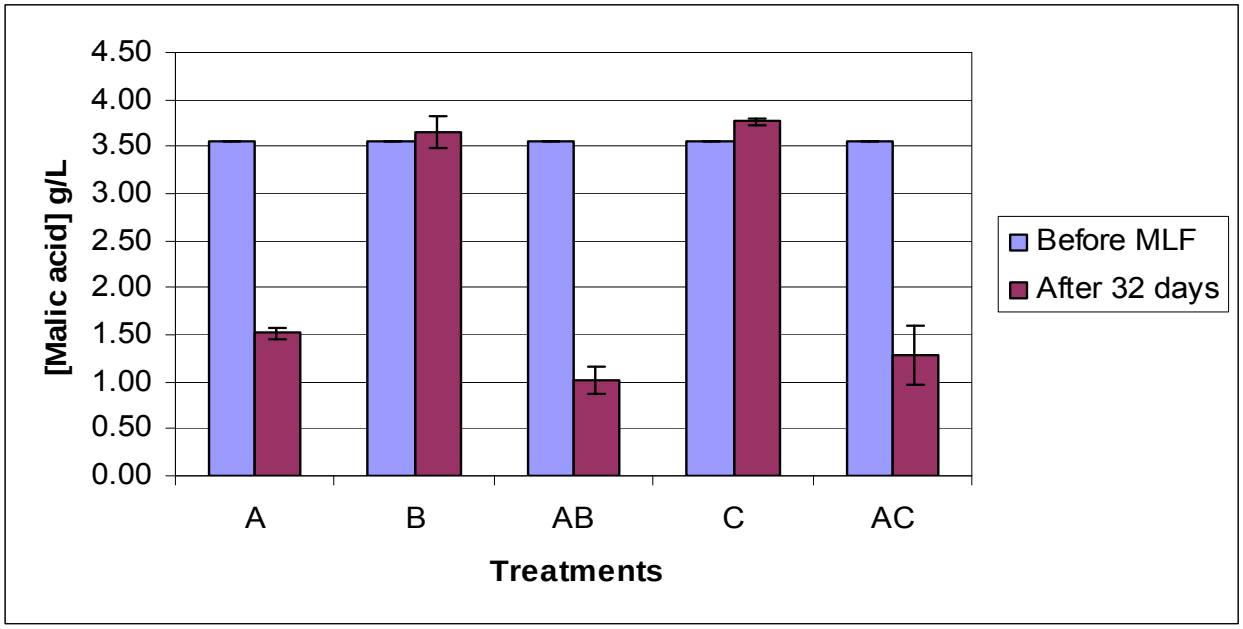


**Figure 4.2** Malic acid degradation for all the treatments measured before MLF and after MLF. A represents the sample that was inoculated with CH16; B was inoculated with isolate 16.7; AB is the combination of the organisms A and B; C represents F14 and AC are the combination between organisms A and C. The error bars indicate the standard deviation of the triplicates.

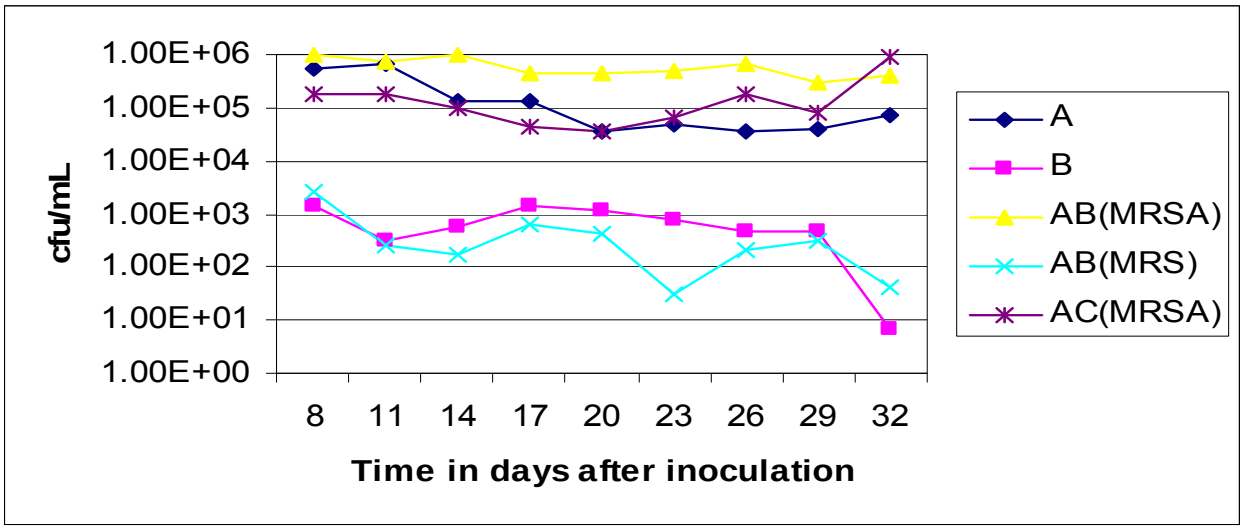


**Figure 4.3** Cell concentrations obtained from the Cabernet Sauvignon pH 4 wine during the course of 32 days for the different treatments. A represent the samples inoculated with CH16; B is the sample set inoculated with isolate 16.7; AB(MRSA) is the combination treatment of CH16 with isolate 16.7 plated on MRS A solid media; AB(MRS) combination treatment of CH16 with isolate 16.7 plated on MRS solid media; C represents the treatment with F14; AC(MRSA) is the cell counts obtained from MRS A media for the combination treatment of CH16 with F14; AC(E) combination treatment of CH16 with F14 plated on MRSetOH solid media. The cell numbers for the acetic acid bacteria are not shown on this figure since no cell numbers were detected.

The biogenic amine levels were measured on day 39 and only putrescine and cadaverine were detected (histamine and tyramine levels were also assessed). In the case of putrescine (**Figure 4.4**) and cadaverine (**Figure 4.5**) levels, the highest amount was found in treatments A, followed by B, with AC having the lowest level. Histamine and tyramine are the most toxic BA's, whilst putrescine and cadaverine are important because it intensifies the effects of histamine (Stratton *et al*., 1991). High levels of BA's were often associated with strains of *Lactobacillus* and *Pediococcus*, but recently also *O. oeni* have been found to be capable of BA production (Lonvaud-Funel and Joyeaux, 1994; Lonvaud-Funel, 2001; Guerrini *et al.*, 2002; Gardini *et al*., 2005).

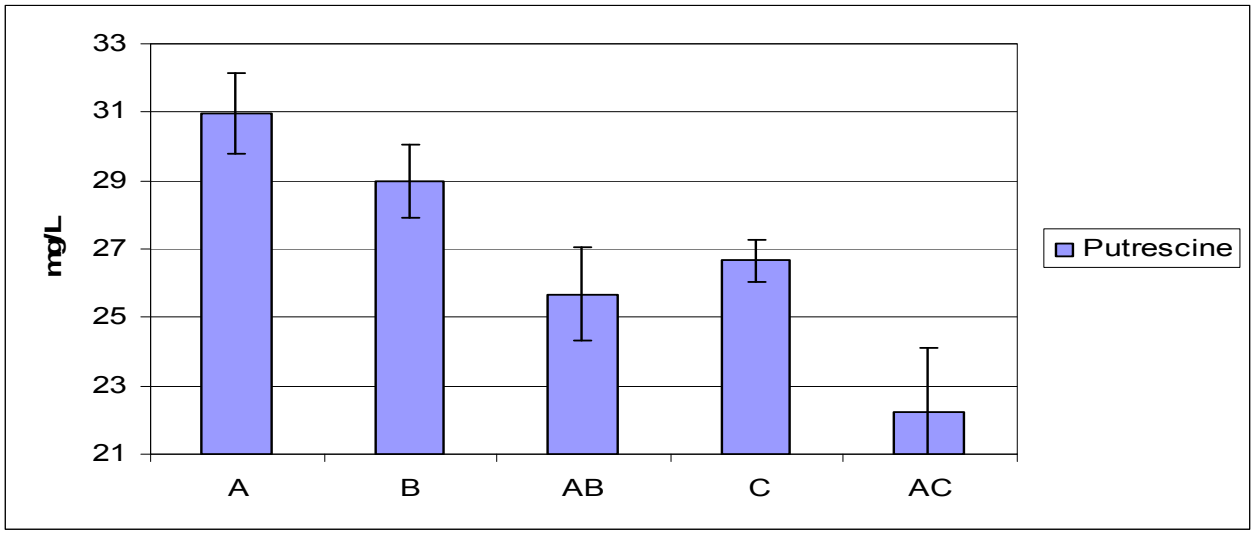


**Figure 4.4** Putrescine in mg/L as measured by HPLC for all the treatments. The error bars represents the standard deviation of the triplicates.

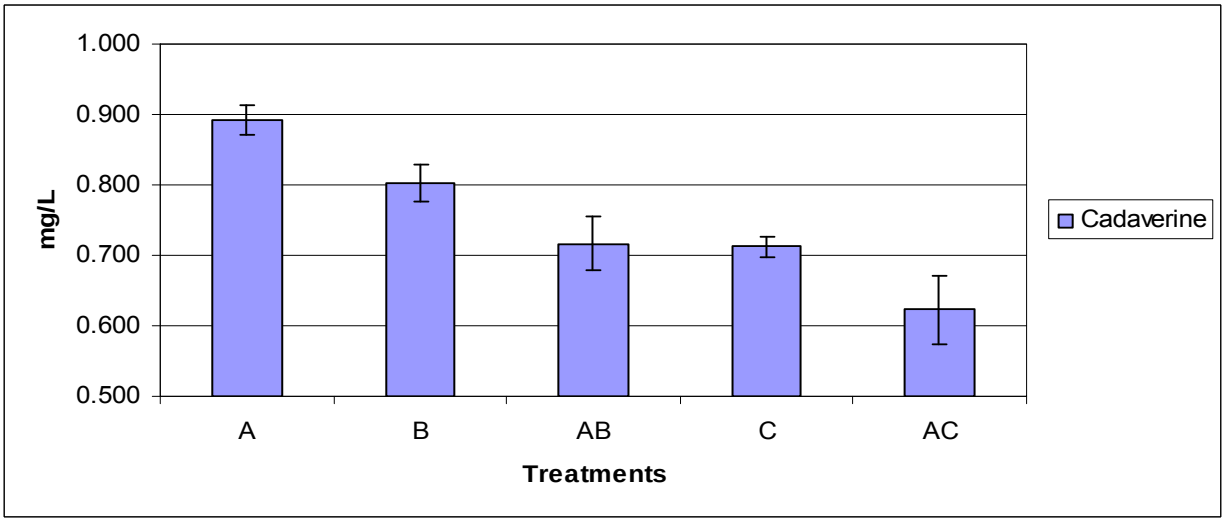


**Figure 4.5** Cadaverine in mg/L as measured by HPLC for all the treatments. The error bars represents the standard deviation of the triplicates.

## 4.4 CONCLUSION

In this experiment possible synergisms between natural LAB and AAB on MLF starter cultures were tested. The results showed a possible synergistic working or activating effect of isolate 16.7 on starter culture CH16 with regard to the degradation of malic acid. This higher rate of malic acid degradation could be due to the accumulative cell concentration of the organisms that were inoculated together, or because of a competition

effect between the organisms with regards to nutrient availability. The only two biogenic amines that were detected after 32 days were putrescine and cadaverine.

Future work could include a more extensive variation of natural occurring LAB inoculated in combination with more commercial starter cultures. The pH range could also be altered, since the pH 3.0 wines were maybe too harsh conditions for the survival of the bacteria.

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# 5. RESEARCH RESULTS

# The effect of commercial tannins and a pectolytic enzyme on malolactic fermentation and phenolic composition of red wine

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## ABSTRACT

Commercial tannins and pectolytic enzymes are added to wine, amongst various other outcomes, to increase the total amount of phenolic compounds in wine as well as to aid colour stability.

The effect of four commercial tannins (condensed, hydrolysable and a mixture) and a pectolytic enzyme on malolactic fermentation induced by three different starter cultures was evaluated in Pinotage and Merlot wines. This was done to evaluate the possible affect these additions might have on the rate of MLF as well as the phenolic constitution of the wine. The malic acid degradation was monitored weekly from the inoculation of MLF. After AF and after MLF the colour density, total phenols, total anthocyanins and total tannins were also measured. For the Pinotage wine the biogenic amine levels was measured after MLF. The results showed a slight inhibitory effect, especially in the first week of MLF, of the tannins on the MLF starter cultures. The colour density increased in the Pinotage due to the addition of tartaric acid after AF, whilst a decrease was found in the Merlot over the period from AF until the end of MLF. The other phenolic measurements showed a decrease from AF to MLF in most cases.

## 5.1 INTRODUCTION

Tannins are phenolic compounds which are of great importance to wine. Tannins are added to wine for the following purposes or corrections: as a redox buffer; sun-damaged fruit; unripe grape tannins; structural/textural; mouth feel modification; increased substrate for micro-oxidation; limit the activity of laccase; assist to precipitate proteins; help to modify

aromas, including vegetative aromas; help increase ageing potential and stabilize red wine colour (Zoecklein, 2005). Legislation exists with regards to tannin additions which differ with respect to countries. Another manner which can be employed to increase the phenolic extraction (especially tannins and colour compounds) in wines is the addition of pectolytic enzymes.

Two groups of tannins excist namely: 1) condensed tannins (also known as proanthocyanidins) which are derived from grapes and 2) hydrolysable tannins that are present in oak and nutgalls. Commercial tannins are classified as derived from grapes when the total flavanol content expressed as (+) catechin, is > 50 mg/g or when its proanthocyanic tannin content is > 0.5 mg/g. Exogenous tannin has its origin from nutgall when the digallic acid content is between 4-8 mg/g and its origin from oak when the scopoletine content is > 4 µg/g (Solich *et al*., 1995; Resolution Oeno, 2002).

Condensed tannins consist of polymerized flavanol units. Flavanol units consist of catechin, epicatechin, gallocatechin, epigallocatechin and epicatechin gallate (Prieur *et al*., 1994; Souquet *et al*., 1996). Tannins are classified either as procyanidins or as prodelphinidins. Procyanidins are catechin- and epicatechin based polymers, while prodelphinidin also contain gallocatechin- and epigallocatechin units in addition to catechin and epicatechin (Hagerman, 2002). The flavonol units can polymerise through either direct polymerisation (Allen *et al*., 1997; Prieur *et al*., 1994; Ribéreau-Gayon *et al*., 1998) or indirect polymerization (Drinkine *et al*., 2005; Fulcrand *et al*., 1997; Ribéreau-Gayon *et al*., 1998; Vidal *et al*., 2004). Condensed tannins change in concentration and structure with grape maturity and also vary according to cultivar (Oberholster, 2003). The highest levels are found to be just before vérasion after which it decreases through *veraison* up to harvesting (Harbertson *et al*., 2002; Oberholster, 2003). Between 1-4 g/L proanthocyanidins are normally extracted from the grapes during fermentation (Ribéreau-Gayon *et al.*, 1998). The extraction of tannins during fermentation depends on the technology used during winemaking, for instance vigorous crushing, pump-overs, punch downs, cold soaking and higher maceration temperatures can increase the extraction (Oberholster, 2003; Sun *et al*., 1999). Condensed tannins play a role in the major organoleptic properties of wine namely astringency, browning and turbidity (Ricardo-da-Silva *et al*., 1993).

Hydrolysable tannins consist of a polyhydric alcohol (typically based on D-glucose) as a basic structural unit of which the hydroxyl groups have been esterified by gallic acid or

hexahydroxydiphenic (HHDP) acid (Hagerman, 2002; Hagerman and Butler, 1991). These tannins can be hydrolysed in either acid or base conditions or enzymatically to yield free gallic acid or HHDP, while the latter can spontaneously hydrolyse to yield ellagic acid. Hydrolysable tannins can be classified as either gallotannins or ellagitannins, according to the type of acid formed (Puech *et al*., 1999). Ellagitannins are extracted from wood (Puech, *et al*., 1999) while gallotannins are extracted from nutgalls (from chest nut trees) and can be added to wine in the form of commercial tannin additions (Hagerman, 2002).

Investigations into the effect of commercial tannin additions to wine have not yet been extensively studied, but the effects of condensed tannin addition and hydrolysable tannin additions on its own in wine have been researched (Pocock *et al*., 1994; Puech *et al*., 1999; Quinn and Singleton, 1985; Vidal *et al*., 2004).

Malolactic fermentation (MLF) is one of the two important fermentations during the winemaking process that could be affected by the addition of commercial tannins. MLF is most of the time obligatory in the production process of red wine, which contains a large range of phenolic compounds. In 1970, Beelman and Gallander, conducted an experiment where MLF was induced in grape must prepared by cold pressing, hot pressing and fermentation on the skins for 1, 3 and 5 days before pressing. The results revealed that fermentation on the skins had a profound effect on MLF. MLF was completed only in the 5 day treatment with fermentation on the skins. They concluded that skin contact must have stimulated the growth of the MLF bacteria.

Later studies were mostly done with the phenolic acids and their potential influence on MLF. Vivas *et al*. (1996) found that gallic acid had an activating effect on cell growth and rate of fermentation of *Oenococcus oeni*, while vanillic acid was slightly inhibitory, which was also confirmed by Lonvaud-Funel (2001) (both benzoic acids). The cinnamic acids all decreased the growth yield of *O. oeni* and *Lactobacillus plantarum* (Salih *et al*., 2000). Another study by Alberto *et al*. (2001) showed that gallic acid activated the rate of glucose and fructose utilization and that the gallic acid was consumed from the beginning of the growth of *L*. *hilgardii*. Therefore gallic acid could potentially increase the formation of spoilage compounds in the presence of *Lactobacillus hilgardii*. Campos *et al*. (2003) monitored an ethanol containing medium supplemented with varying concentrations of hydroxybenzoic and hydroxycinnamic acids. It was found that the hydroxycinnamic acid was more inhibitory to *O*. *oeni* than the hydroxybenzoic acids (gallic and vanillic acid). The hydroxycinnamic acids (caffeic and ferulic acid), were more beneficial to the growth of

*L*. *hilgardii*. *p*-Coumaric acid had the strongest inhibitory effect on the growth and survival of both bacterial species. Hydroxycinnamic acids have also been found to have an inhibitory effect on *O*. *oeni* at high concentrations (Reguant *et al*., 2000). They also found that catechin and quercetin (flavonoids) stimulated MLF but delayed or inhibited the formation of acetic acid from citric acid. This could potentially suppress the increase in volatile acidity (VA). Catechin also stimulated MLF (measured as malic acid consumption) for *L*. *hilgardii* (Alberto *et al*., 2001). Vivas *et al*. (1997) also studied the effect of anthocyanins on the growth of *O*. *oeni* and the rate of malic acid degradation and found that it activated both processes.

This study focussed on the effect of the addition of commercial tannins (condensed tannins, hydrolysable tannins and a combination) as well as a pectolytic enzyme on the performance of commercial MLF starter cultures. Their influence on the malic acid degradation rate was be determined. The colour density, total phenols, total anthocyanins and total tannins were also measured before and after MLF to determine if differences in these parameters occurred between the different tannin/enzyme treatments and the different starter culture strains.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Experimental layout

This study included four different commercial oenological tannin preparations as well as a pectolytic enzyme. The methodology of the layout of the experiment is denoted in **Table 5.1**. **A** represents the duplicate samples that were prepared from the wine after AF with a single dosage of tannin/enzyme. **B** represents the duplicate samples that were prepared from the wine after AF by adding another dosage of tannin/enzyme to create a double dosage of tannin, whilst **C** represents the duplicate samples prepared by adding a double dosage of tannin to the wine after AF, to establish a triple dosage of tannin.

**Table 5.1** The experimental outline for this study

**Homogenised must of Pinotage and Merlot**

Alcoholic fermentation

Add a single dosage of the respective tannin/enzyme to each fermenter at beginning of AF (except control)

| Control **(C)** | Taniflora **(T)** | Oenotan **(Oe)** | QCTN **(Q)** | VR Supra **(V)** | Lafase HE **(L)** | |
|---|---|---|---|---|---|---|
| Malolactic fermentation | | | | | | |
| Viniflora Oenos **(O)*** Triplicate | **O*** Triplicate | **16*** Duplicate | **16*** Duplicate | **16*** Duplicate | **16*** Duplicate | **A** |
| Viniflora CH16 **(16)*** Triplicate | **16*** Triplicate | **16** **+ Oe** Duplicate | **16** **+ Q** Duplicate | **16** **+ V** Duplicate | | **B** |
| Viniflora CH35 **(35)*** Triplicate | **35*** Triplicate | **16*** **+ 2 X Oe** Duplicate | **16*** **+ 2 X Q** Duplicate | **16*** **+ 2 X V** Duplicate | | **C** |

* inoculate wine for MLF after AF

** inoculate wine for MLF after adding another dosage of the respective tannin/enzyme

*** inoculate wine for MLF after adding a double dosage of the respective tannin/enzyme

The tannins and the enzyme were added at AF, as prescribed by the respective suppliers. The tannins used and maximum dosages as recommended by the suppliers are listed in **Table 5.2**.

**Table 5.2: The type of commercial tannins/enzyme and the dosages at which it was added during AF for the Pinotage and Merlot.**

| Treatment and commercial source | Type of tannin/enzyme according to the supplier | Dosage (mg/L) |
|---|---|---|
| Lafase HE Grant Cru (L) (Laffort Œnologie) | Pectolytic enzyme | 50 |
| Oenotan (Oe) (Colombit) | Hydrolysable | 250 |
| QCTN (Q) (Warren chem.) | Hydrolysable | 500 |
| Tanin VR Supra (V) (Laffort Œnologie) | Condensed and Hydrolysable | 500 |
| Taniflora (T) (ChrHansen) | Condensed | 250 |

### 5.2.2 Preparation of must

Pinotage and Merlot grapes from the Stellenbosch region (Western Cape, South Africa) were used to produce wines for this study during the 2006 season. The grapes were destemmed and crushed and 30 ppm $SO_2$ was added to the must. The grapes were homogenised and the juice and skins were separated after crushing before they were divided equally into 6 fermenters. A fermenter consisted of 40 L plastic bins with a lid. Approximately 40 kg skins and 18 L of free running juice were put into each fermenter. The pH and titratable acid (TA) were determined using a Metrohm titration unit (Metrohm Ltd. Switzerland) as well as the °Brix was measured with a balanced hydrometer.

### 5.2.3 Wine fermentations

The must in each of the six fermenters was inoculated with WE372 (Anchor Yeast Biotechnologies, South Africa) wine yeast at 30 g/hL. Fermentation was conducted in a controlled fermentation room which were kept at approximately 25˚C. Punch downs were done twice daily and the sugar content was measured once a day with a hydrometer. Approximately 3 days in AF, di-ammonium-phosphate (DAP) was added at 50 g/hL as a yeast nutrient. The wines were fermented on the skins till dry (less than 5 g/L residual sugar) and then pressed. The wine from each fermenter was respectively pressed and mixed before it was aliquoted into 2 L glass bottles. This was done in triplicate for the control and Taniflora treatments, while the other treatments were performed in duplicate. The additional tannin treatments were added at this point (after pressing, before inoculation for MLF) to the wine and then the wines were inoculated for MLF. MLF was conducted at 20˚C.

Three different starter culture was used namely, Viniflora®Oenos, Viniflora®CH16 and Viniflora®CH35 (ChrHansen, Denmark). These cultures consist of *O. oeni*. The cultures were inoculated into the wines, at a rate of greater than 5 x $10^6$ cfu/mL, as prescribed by the supplier.

### 5.2.4 Media and culture conditions

The must and wine were plated on MRS agar (Biolab, Merck, South Africa) enriched with 20% apple juice (containing no preservatives) (MRSA) (pH5.2). ). The addition of apple juice and lowering of the pH stimulates the growth of *O*. *oeni*. Normal De Man, Rogosa and Sharpe (MRS) agar was also used for the enumeration of LAB. Both MRSA and MRS

contained 100 mg/L Actistab (50% glucose, 50% natamycin, Gist-brocades, France, S.A.), dissolved in methanol, for the inhibition of moulds, fungi and yeast. Kanamycin sulphate ($C_{18}H_{36}N_4O_{11}$ X $H_2SO_4$, Roche Diagnostics) at 25 mg/L dissolved in distilled water, were used for the inhibition of acetic acid bacteria (AAB).

Yeast Peptone Dextrose (YPD) agar (Merck, Biolab Diagnostics (Pty) Ltd) was used for the enumeration of yeast. Kanamycin sulphate was also used in the YPD media for the inhibition of AAB and 50 mg/L Nisin (Sigma-Aldrich Co., dissolved in methanol) for the inhibition of LAB. MRS medium to which 2% v/v ethanol (MRSetOH) was added, was used for the enumeration of AAB. 100 mg/L Actistab and 50 mg/L Nisin were used to inhibit yeast and LAB respectively. The MRSA and MRS were incubated under facultative anaerobic conditions at 30˚C (Anerogen, Oxoid) in a rectangular anaerobic jar (Davies Diagnostics (Pty) Ltd.), whilst YPD and MRSetOH media was incubated at 30˚C aerobically.

Enumeration was done for the juice at crushing, after AF on the wine and also after MLF. The samples were diluted within a range of $10^{-1}$-$10^{-6}$ depending on the stage of winemaking, using 1 mL of sample and standard test tubes with 9 mL sterile water.

### 5.2.5 Analyses of wine parameters by FT-IR spectroscopy

L-Malic acid was measured weekly from inoculation of the starter culture using Fourier Transformation Infrared Spectroscopy (FT-IR) (Foss Grape scan). Samples were filtered with a Filtration Unit (type 79500, FOSS Electric, Denmark) connected to a vacuum pump. The filter unit uses filter paper circles graded at 20 – 25 µm with diameter 185 mm (Schleicher & Schnell, reference number 10312714). The filtered must were used for FT-IR spectral measurements. A Winescan FT120 equipped with a purpose built Michelson interferometer was used to generate the FT-IR spectra (FOSS Electric A/S, Hillerød, Denmark). Instrument settings included: cell path length of 37 µm, sample temperature set to 40°C, and sample volume of 7 – 8 ml. The sample is pumped through the heat exchanger and the $CaF_2$-lined cuvette. Samples are scanned from 5011 to 926 $cm^{-1}$ at 4 $cm^{-1}$ interval.

Other analysis that was done with FT-IR technology is the monitoring of routine wine parameters (pH, Total acidity (TA), Volatile acidity (VA), Malic acid, Lactic acid, Ethanol, Glucose, Fructose and Glycerol) for the juice after crushing and the wine after AF.

### 5.2.6 Analyses of the phenolic compounds

The colour density, total red pigments, total phenolics, total tannins and total anthocyanins were measured after AF and after MLF for all the treatments in both cultivars. These measurements were done using a Heλios spectrophotometer (Thermo electron corporation Ltd., United Kingdom). Depending on the wavelength of the analysis or density of the wine, 10 mm quartz, 1 mm glass or 10 mm plastic cuvettes were used. The total tannins and total anthocyanins were determined by methods described by Ribéreau-Gayon *et al*. (1998), while the colour density, total red pigments and total phenolics were determined by methods described by Iland *et al*. (2000).

## 5.3 RESULTS AND DISCUSSION

### 5.3.1 Pinotage 2006

#### 5.3.1.1 Malic acid degradation

The malic acid reduction rate for the controls (no tannins added) with the three different starter cultures (CO, C16 and C35) was compared to the treatments that received taniflora commercial tannin (T) at alcoholic fermentation (single dosage of 250 mg/L) with the three different starter cultures (TO, T16, T35) (**Figure 5.1**). After week 1 of malolactic fermentation the reduction rate was 74% for CO compared to the 60% reduction in TO. The same trend was found for the C16 and T16 samples with a reduction rate of 74% and 67% respectively in week 1. This indicates that Taniflora had no significant or major impact on the malolactic fermentation of these two MLF strains. The control and treatment inoculated with Viniflora®CH35 (C35 and T35) had a slow initiation of MLF during week 1 and 2. This could be explained by the fact that Viniflora®CH35 is a starter culture that was isolated and produced for use in white grape cultivars by the suppliers and therefore it would find the more complex matrix of red wine as harsh conditions compared to the other two starter cultures. At week 1 no malic acid reduction was observed in C35 while T35 reduced it by 6%. At week 2, C35 (control without tannin) had a reduction rate of 55%, while the treatment with Taniflora (T35) only had a 31% reduction. This could be due to the initial inhibition of Taniflora on this starter culture. At week 3 the malic acid degradation was more or less the same between C35 and T35 and also in the same range as for the other treatments. The cell numbers after MLF varied between 4.28 x $10^5$ and

2.13 x $10^6$ cfu/mL for these treatments, levels which could be expected after MLF (**Figure 5.2**). The C35 cell numbers were lower than the C16 and CO, which correlated with the slower rate of MLF.

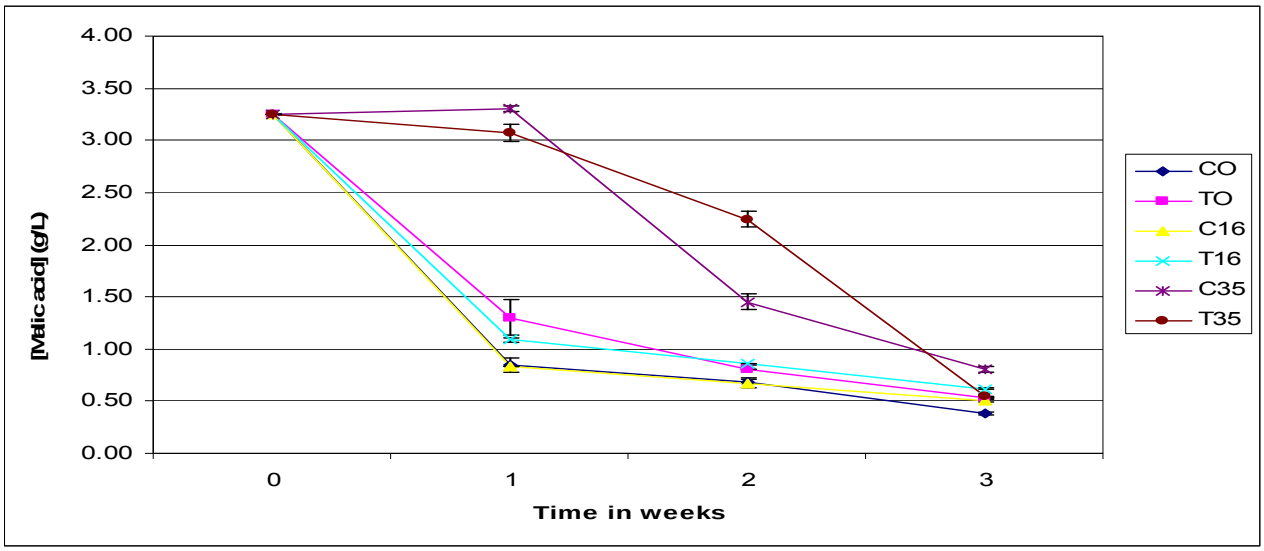


**Figure 5.1** Degradation of malic acid monitored during MLF in Pinotage 2006 for the controls (C) and treatments with Taniflora (T). Taniflora was added to the wine at a concentration of 250 mg/L at fermentation. MLF was inoculated at 1 g/hL with three different commercial starter cultures: Viniflora®oenos (O), Viniflora®CH16 (16) and Viniflora®CH35 (35). Error bars indicate the standard deviation of the triplicates.

The treatments with Oenotan (Oe), QCTN (Q), VR Supra (V) and the pectolytic enzyme (L), were all inoculated with only Viniflora®CH16 and therefore will be compared only to the control treatment with Viniflora®CH16 (C16).

When comparing the treatments Oenotan (OeA, OeB and OeC) and C16 the results showed that the malic acid degradation rate was proportionally slower with the increase in dosage of the tannin (**Figure 5.3**). Malic acid reduction for C16, OeA, OeB and OeC was 74%, 64%, 55% and 43% respectively in week 1. This indicated than each additional dosage decreased malic acid reduction with approximately 10% over week 1. This inhibition by the tannin was continued over week 2 and 3 but the reduction rate of the three dosage treatments became more or less the same (84% for C16 and 77% for OeA, B and C).

The treatments with tannin QCTN showed a slight inhibition on the MLF starter culture (**Figure 5.4**), but in this case the treatments continued to reduce malic acid at a slower rate over all 3 weeks. The malic acid reduction pattern was C16>QA>QB>QC, which indicated that the higher the dosage of tannin the lower the malic acid reduction rate. The largest difference in malic acid degradation rate was found between C16 and QC, which

was 74% and 65% respectively in week 1, 79% and 67% respectively in week 2 and 84% and 74% respectively in week 3. A constant difference of approximately 10% in the reduction rate was therefore maintained throughout MLF between the control and triple dosage treatment of QCTN. The cell numbers obtained at the end of MLF showed no differences between the treatments (**Figure 5.2**).

Oenotan and QCTN are hydrolysable tannins as promoted by the suppliers, which means that the building blocks are the non-flavanoids or hydrocinnamic acids and hydrobenzoic acids. Various studies have been done on the effects of these acids (in the pure form) on the growth of LAB and more specifically *O. oeni*. Gallic acid was found to have an activating effect on cell growth and rate of fermentation of *O. oeni*, while vanillic acid was slightly inhibitory (Lonvaud-Funel, 2001; Vivas *et al*., 1996). Hydroxycinnamic acids are more inhibitory to *O. oeni* than the hydroxybenzoic acids (Compos *et al*., 2003; Reguant *et al*., 2000; Salih *et al*., 2000). The exact constitution of these commercial hydrolysable tannins is not known, but since a slight inhibitory effect of the tannins on the reduction rate of malic acid was found during this study, it could be speculated that it consists of more hydrocinnamic acids. It could also be that the concentration of actual tannins in these products could be very low.

The treatment with VR Supra commercial tannin (V) showed a slight inhibitory effect on the MLF starter culture for the triple dosage (VC) but only in week 1 (**Figure 5.5**). The reduction of malic acid for C16 was 74% in week 1 whilst the reduction was 58% for VC. The other treatments had more or less the same reduction level that the control and in week 2 and 3 all the treatments had more or less the same reduction level as the control (between 74% and 79% week 2 and between 81% and 84% in week3). VR Supra is a mixture of hydrolysable and condensed tannins according to the supplier. The major components of condensed tannins are flavan-3-ols and flavan-3,4-dioles. In a study by Vivas *et al*. (1997) anthocyanins activated the growth of *O*. *oeni* and the rate of malic acid degradation. As mentioned previously, the full constitution of this product is not known and therefore also not the ratio of hydrolysable tannin vs condensed tannin. The treatment with Lafase (pectolytic enzyme) had no effect on the degradation of malic acid when compared to the control sample (**Figure 5.6**).

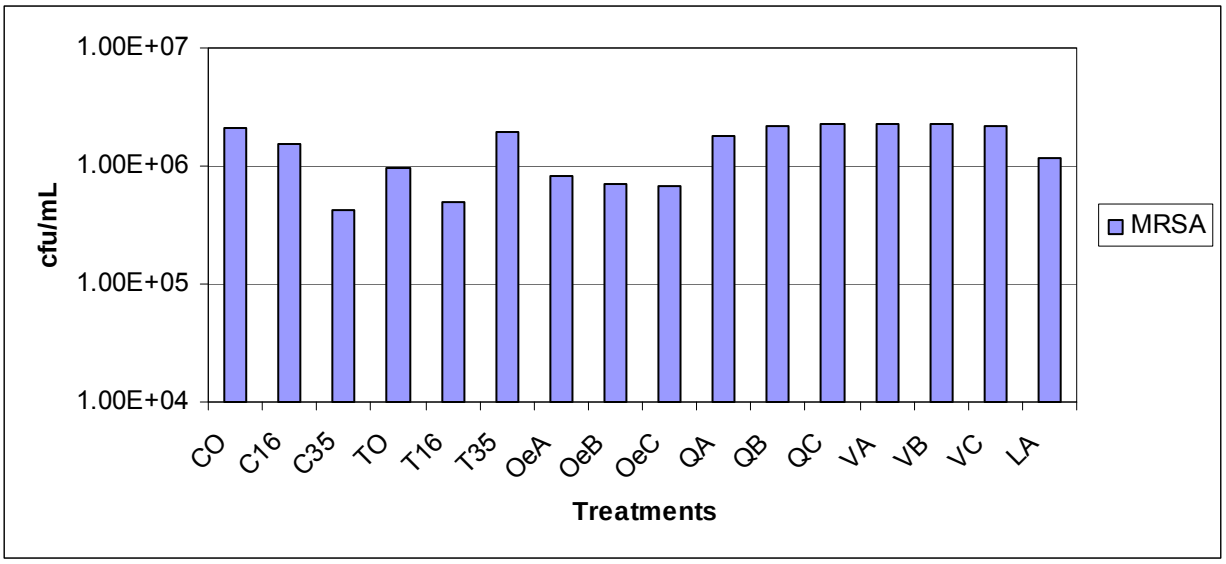


**Figure 5.2** Cell numbers obtained after MLF for Pinotage 2006 wine for all the treatments. C represents the control treatments and T represents the Taniflora treatment. MLF was inoculated at 1.5 g/250 L with three different commercial starter cultures: Viniflora®oenos (O), Viniflora®CH16 (16) and Viniflora®CH35 (35) in these two treeatments. Oe represents sample to which Oenotan was added; Q represents samples with QCTN added; V represent the samples to which VR Supra was added and L represents samples to which lafase pectolytic enzyme was added. A represents a single dosage of tannin (at AF); B represents a double dosage of tannin and C represents a triple dosage of tannin after AF before MLF. These samples were all inoculated only with with Viniflora®CH16 (16) for MLF. Each bar indicates the average of the repeats.

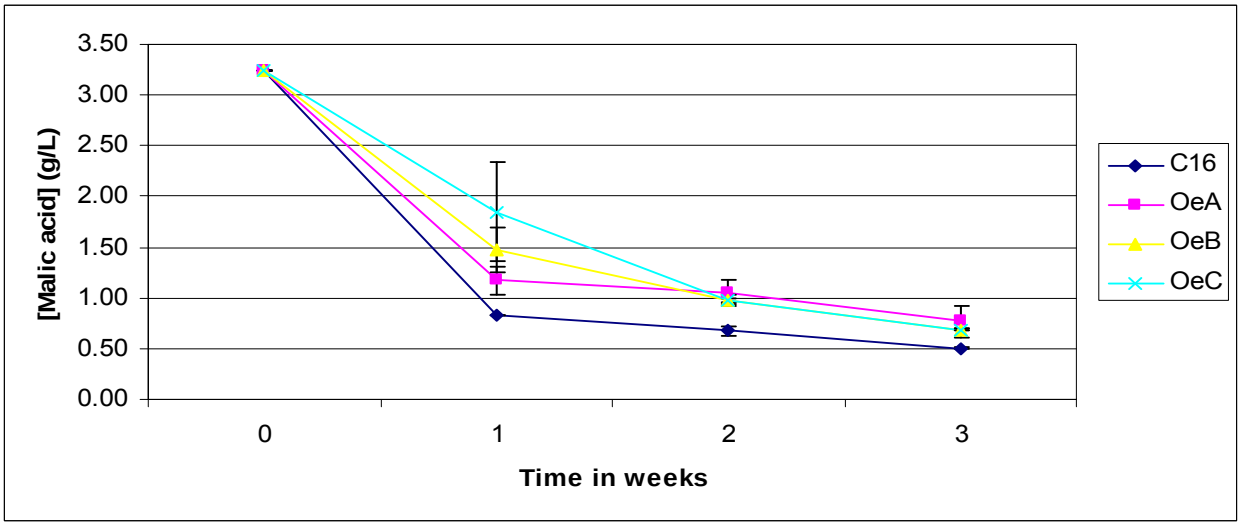


**Figure 5.3** Degradation of malic acid monitored during MLF in Pinotage 2006 for the control with (C) and treatments with Oenotan (Oe). Oenotan was added to the wine at a concentration of 250 mg/L, treatment included a single dosage of tannin (at AF) (A) and a double dosage of tannin (B) and triple dosage of tannin (C) after AF before MLF. MLF was inoculated at 1 g/hL with Viniflora®CH16 (16) in the control and treatment. Error bars indicate the standard deviation of the repeats.

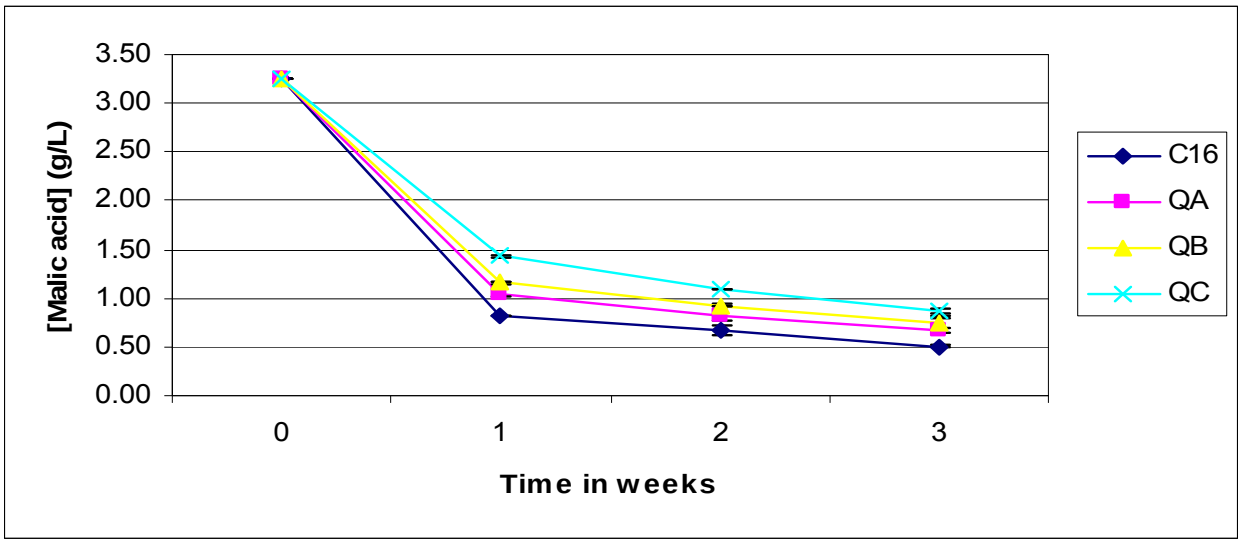


**Figure 5.4** Degradation of malic acid monitored during MLF in Pinotage 2006 for the control with (C) and treatments with QCTN (Q). QCTN was added to the wine at a concentration of 500 mg/L, treatment included a single dosage of tannin (at AF) (A) and a double dosage of tannin (B) and triple dosage of tannin (C) after AF before MLF. MLF was inoculated at 1 g/hL with Viniflora®CH16 (16) in the control and treatment. Error bars indicate the standard deviation of the repeats.

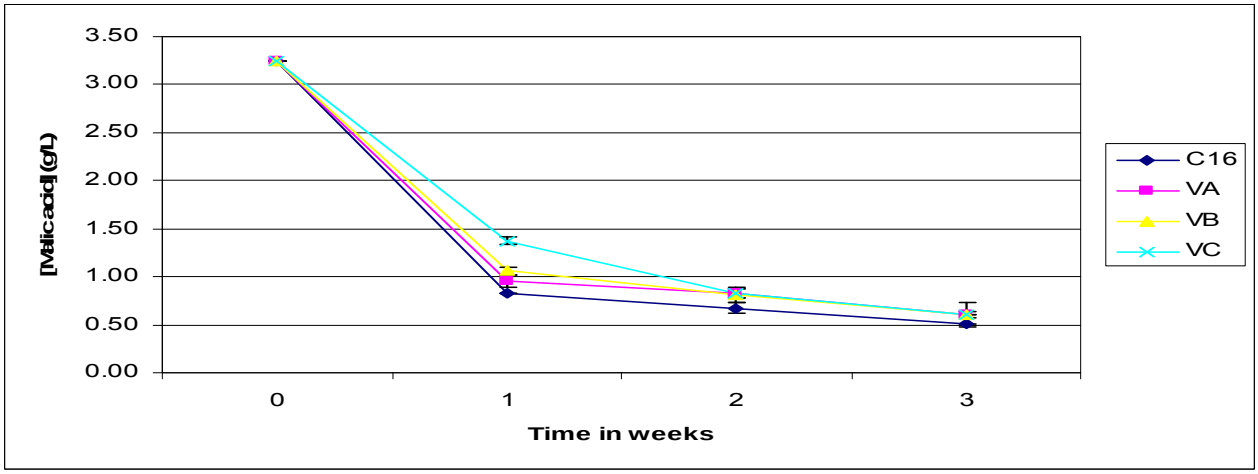


**Figure 5.5** Degradation of malic acid monitored during MLF in Pinotage 2006 for the control with (C) and treatments with VR Supra (V). VR Supra was added to the wine at a concentration of 500 mg/L, treatment included a single dosage of tannin (at AF) (A) and a double dosage of tannin (B) and triple dosage of tannin (C) after AF before MLF. MLF was inoculated at 1 g/hL with Viniflora®CH16 (16) in the control and treatment. Error bars indicate the standard deviation of the repeats.

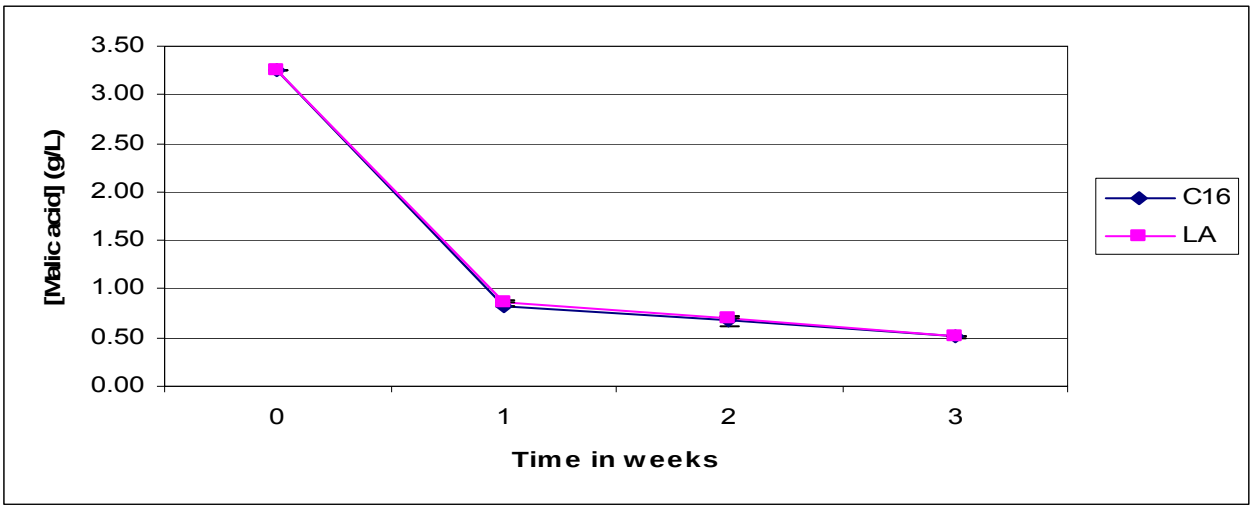


**Figure 5.6** Degradation of malic acid monitored during MLF in Pinotage 2006 for the control with (C) and treatment with Lafase pectolytic enzyme (L). Lafase was added to the wine at a concentration of 50 mg/L at AF in a single dosage (A). MLF was inoculated at 1 g/hL with Viniflora®CH16 (16) in the control and treatment. Error bars indicate the standard deviation of the repeats.

### 5.3.2 Merlot 2006

#### 5.3.2.1 Malic acid degradation

The degradation of malic acid in the Merlot wines did not show any large difference between most of the treatments (T, Oe and L) and the controls C. During week 1 for most of these treatments the reduction percentage of malic acid was between 2% and 10%. Compared to the Pinotage, merlot had very little malic acid degradation in the first week. This similar level of reduction continued throughout MLF for these samples.

For the treatment with QCTN (**Figure 5.7**) the reduction of malic acid developed at approximately the same rate, but at week 4 no malic acid was detected for the treatment QB and QC, but the control had a reduction of 97% at this stage, which indicates that MLF was technically completed for these samples as well.

In the VR Supra treatments (VA, VB and VC) the malic acid reduction at week 1 showed a 3% reduction in the control (C16), but a reduction of 50% for treatments with this tannin (**Figure 5.8**). VR Supra is a mixture of hydrolysable and condensed tannins. Therefore this mixture could contain flavanoids as well as non-flavonoid phenols (Ribéreau-Gayon *et al*., 1998). Compounds that have been found to stimulate the growth of LAB are for instance gallic acid, anthocyanins and vannilin (De Revel *et al*., 2004; Vivas *et al*., 1995). At week 2 and 3 the reduction percentages were more or less similar, but in week 4 no malic acid was detected in the VA and VC treatments. This could indicate that VR Supra stimulated the malic acid degradation, which was not found in the case of the

Pinotage. From the plating out of the wine on MRS and MRS A media cell concentrations were found to be at the same level of approximately $10^6$ cfu/mL for all the treatments. The growth on MRS could also be *Oenococcus. oeni* since they are also able to grow on MRS.

No AAB was detected on the MRSEtOH media throughout the experiment and yeast cell numbers were only obtained for the juice after crushing and therefore are not shown.

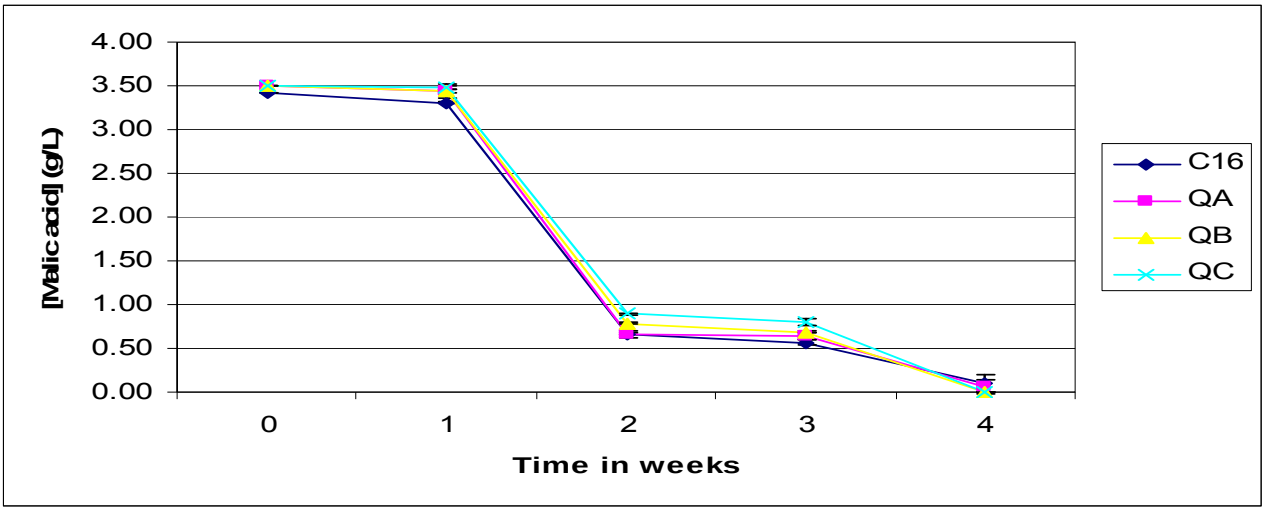


**Figure 5.7** Degradation of malic acid monitored during MLF in Merlot 2006 for the control with (C) and treatments with QCTN (Q). QCTN was added to the wine at a concentration of 500 mg/L, treatment included a single dosage of tannin (at AF) (A) and a double dosage of tannin (B) and triple dosage of tannin (C) after AF before MLF. MLF was inoculated at 1 g/hL with Viniflora®CH16 (16) in the control and treatment. No malic acid detected for QB and QC in week 4. Error bars indicate the standard deviation of the repeats.

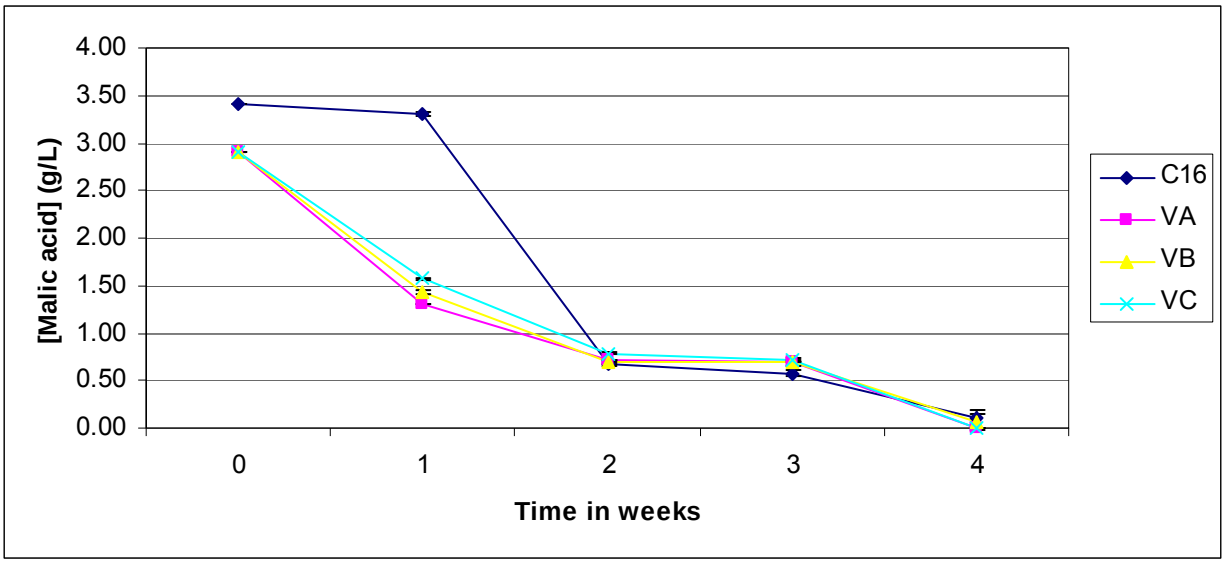


**Figure 5.8** Degradation of malic acid monitored during MLF in Merlot 2006 for the control with (C) and treatments with VR Supra (V). VR Supra was added to the wine at a concentration of 500 mg/L, treatment included a single dosage of tannin (at AF) (A) and a double dosage of tannin (B) and triple dosage of tannin (C) after AF before MLF. MLF was inoculated at 1 g/hL with Viniflora®CH16 (16) in the control and treatment. Error bars indicate the standard deviation of the repeats.

### 5.3.3 Pinotage and Merlot 2006

#### 5.3.3.1 Colour density

During this study the CD was measured at the end of AF and then again at the end of MLF. After alcoholic fermentation the highest CD level in the Pinotage wine was found in the fermentation with a single dosage of Taniflora, whilst the lowest CD level was found in the Oenotan fermentation (**Table 5.3**). Taniflora is a condensed tannin, while Oenotan is a hydrolysable tannin. Kovac *et al*. (1992, 1995) found that the addition of 60 g seeds per kg of grapes resulted in an increase in colour density, which collaborates with the higher CD in the Taniflora treatments. During MLF an increase in CD was observed in the Pinotage wine for almost all the treatments except for the control with Viniflora®CH35 (C35) and the treatment with Taniflora and Viniflora®oenos (TO) (**Table 5.3**). In the case of the Merlot wine the CD was found to decrease for almost all of the treatments except for the control with Viniflora®CH35 (C35) where a slight increase was observed (**Table 5.4**). In a study by Keulder (2005) where commercial tannins were added to Shiraz and Merlot wines a decrease in CD was found from AF to MLF. This collaborates with the Merlot data found in this study. The reason for the decrease in CD during MLF is strongly related to the pH increase as a result of MLF, the red flavylium kation changes to the quinonic base form which is blue with an increase in pH (Ribéreau-Gayon *et al*., 2000). This is further

collaborated by the fact that for the Merlot wine the red colour of all of the treatments (measured at 520nm) decreased, whilst the purple colour (measured at 620nm) increased from AF to the end of MLF (**Table 5.4**). The greatest decrease in CD in the Merlot wine was found in the treatments with QCTN followed by the treatments with VR Supra and Lafase treatment. Another reason for the decrease in red colour could be the polymerisation and precipitation of the anthocyanins (Gonzalez-Nevez *et al*., 2004; Monagas *et al*., 2006).

**Table 5.3: Colour density (CD) measured at 420nm, 520nm and 620nm after AF and after MLF for Pinotage 2006.**

| | After AF | | | | | After MLF | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| ID | 420nm | 520nm | 620nm | CD | ID | 420nm | 520nm | 620nm | CD | % increase/ decrease |
| **C** | 0.364 | 0.594 | 0.185 | 11.4 | **CO** | 0.384 | 0.549 | 0.156 | 13.11 | 12.81 |
| | | | | | **C16** | 0.432 | 0.679 | 0.200 | 13.11 | 12.81 |
| | | | | | **C35** | 0.350 | 0.445 | 0.132 | 9.28 | 23.23 |
| **T** | 0.404 | 0.646 | 0.175 | 12.3 | **TO** | 0.393 | 0.560 | 0.161 | 11.13 | 10.05 |
| | | | | | **T16** | 0.436 | 0.669 | 0.237 | 13.41 | 8.68 |
| | | | | | **T35** | 0.490 | 0.715 | 0.275 | 14.80 | 17.22 |
| **Oe** | 0.299 | 0.486 | 0.128 | 9.1 | **OeA** | 0.479 | 0.707 | 0.300 | 14.86 | 38.57 |
| | | | | | **OeB** | 0.394 | 0.595 | 0.223 | 12.12 | 24.66 |
| | | | | | **OeC** | 0.491 | 0.714 | 0.287 | 14.92 | 38.80 |
| **Q** | 0.338 | 0.513 | 0.145 | 10.0 | **QA** | 0.602 | 0.826 | 0.353 | 17.81 | 44.09 |
| | | | | | **QB** | 0.552 | 0.746 | 0.303 | 16.01 | 37.81 |
| | | | | | **QC** | 0.652 | 0.837 | 0.386 | 18.75 | 46.87 |
| **V** | 0.361 | 0.549 | 0.157 | 10.7 | **VA** | 0.498 | 0.698 | 0.258 | 14.54 | 26.61 |
| | | | | | **VB** | 0.557 | 0.777 | 0.326 | 16.60 | 35.72 |
| | | | | | **VC** | 0.644 | 0.885 | 0.376 | 19.05 | 43.99 |
| **L** | 0.333 | 0.522 | 0.139 | 9.9 | **LA** | 0.438 | 0.626 | 0.191 | 12.55 | 20.77 |

The increase in CD for the Pinotage wine was due to of an increase in the red colour (520 nm) for most of the treatments except for CO, C35 and TO (**Table 5.4**). This increase in red colour could be due to the fact that the pH of the Pinotage was lowered during MLF by tartaric acid addition from pH 4.4 to 3.9. The greatest increase in CD for the Pinotage

wines was found in treatments OeC, QC and VC where a triple dosage of tannins was added.

**Table 5.4: Colour density (CD) measured at 420nm, 520nm and 620nm after AF and after MLF for Merlot 2006.**

| | After AF | | | | | After MLF | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| ID | 420nm | 520nm | 620nm | CD | ID | 420nm | 520nm | 620nm | CD | % Decrease/ increase |
| **C** | 0.584 | 1.185 | 0.190 | 19.59 | **CO** | 0.582 | 0.813 | 0.257 | 16.52 | 15.67 |
| | | | | | **C16** | 0.640 | 0.890 | 0.306 | 18.37 | 6.25 |
| | | | | | **C35** | 0.685 | 0.929 | 0.412 | 20.25 | 3.38 |
| **T** | 0.616 | 1.252 | 0.204 | 20.72 | **TO** | 0.692 | 0.936 | 0.334 | 19.62 | 5.33 |
| | | | | | **T16** | 0.637 | 0.933 | 0.301 | 18.72 | 9.68 |
| | | | | | **T35** | 0.635 | 0.931 | 0.290 | 18.56 | 10.42 |
| **Oe** | 0.581 | 1.193 | 0.189 | 19.63 | **OeA** | 0.603 | 0.916 | 0.294 | 18.13 | 7.62 |
| | | | | | **OeB** | 0.606 | 0.882 | 0.305 | 17.92 | 8.71 |
| | | | | | **OeC** | 0.599 | 0.866 | 0.298 | 17.63 | 10.17 |
| **Q** | 0.689 | 1.315 | 0.253 | 22.57 | **QA** | 0.546 | 0.819 | 0.224 | 15.88 | 29.65 |
| | | | | | **QB** | 0.563 | 0.809 | 0.227 | 15.99 | 29.14 |
| | | | | | **QC** | 0.582 | 0.793 | 0.249 | 16.23 | 28.11 |
| **V** | 0.581 | 1.187 | 0.187 | 19.55 | **VA** | 0.534 | 0.806 | 0.256 | 15.96 | 18.35 |
| | | | | | **VB** | 0.534 | 0.796 | 0.267 | 15.97 | 18.29 |
| | | | | | **VC** | 0.517 | 0.763 | 0.266 | 15.46 | 20.93 |
| **L** | 0.630 | 1.276 | 0.236 | 21.42 | **LA** | 0.596 | 0.874 | 0.315 | 17.85 | 16.68 |

For the Pinotage wine the brown colour increased in almost all cases (measured at 420nm), except for the controls with Viniflora®CH35 (C35) and the treatment with Taniflora tannin and Viniflora®oenos (**Table 5.3**). For the Merlot wine an increase in brown colour was found in the controls and treatment with Taniflora (for all three starter cultures), while in the case of the QCTN, VR Supra and Lafase treatments a decrease was observed (**Table 5.4**). This increase in brown colour could be the result of oxidation of the phenols in the wine (Castelari *et al*., 2000; Perez-Prieto *et al*., 2003), the formation of xanthylium salts (orange) (Dallas *et al*., 1996; Malien-Aubert *et al*., 2002) and polymerization of anthocyanins with tannins (Castillo-Sanchez *et al*., 2005).

#### 5.3.3.2 Total red pigments

In the measurement of the total red pigments (TRP) the pH are lowered to below 1. In this acidious medium all the anthocyanins are in its coloured, flavylium form. Therefore all the anthocyanins, copigmented anthocyanins and pigments are measured. The total red pigments of all the treatments for the Pinotage and Merlot wine showed a decrease from AF to the end of MLF (**Figures 5.9** and **5.10**). For the Pinotage wine the treatments with Oenotan and QCTN showed a greater loss of red pigment (70%), than the other treatments (< 55%) and in the Merlot wine the Oenotan treatment also had the highest reduction in TRP but in this case the VR Supra and Lafase treatments also had a high reduction level in relation to the other treatments. This loss in red pigments could be the result of oxidation (Gómez-Plaza *et al*., 2004) and also the precipitation of the red pigments (Gil-Munoz *et al*., 1997; Perez-Prieto *et al*., 2003). The loss in red pigment can also be due to their polymerization (Saucier *et al*., 2004) and differences in the extinction coefficients of the newly formed pigments compared to the monomeric anthocyanins (Boulton, 2001). Oenotan and QCTN are hydrolysable tannins (according to the supplier), while VR Supra are a combination of hydrolysable and condensed tannin. According to Vivas *et al*. (1996) hydrolysable tannins or wood tannins oxidise easier than grape derived tannins, leading to enhanced polymerization and possible precipitation of pigments, which was observed in some of the treatments with and increase in dosage.

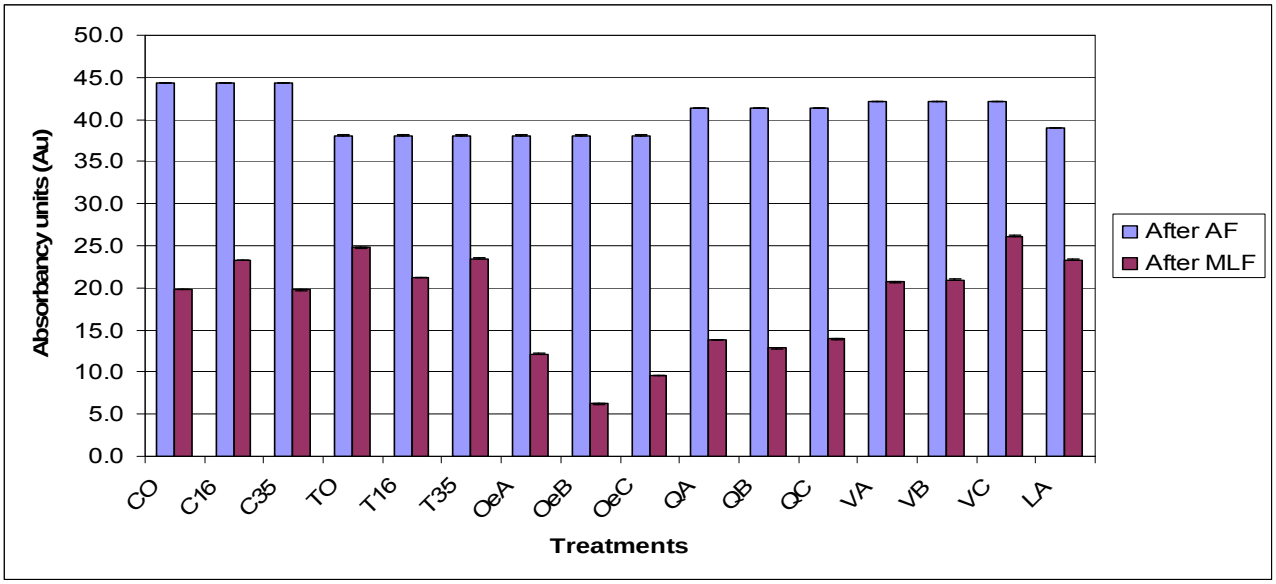


**Figure 5.9** Total red pigments (TRP) after AF and MLF for Pinotage 2006. C represents the controls; T represents the treatment with Taniflora; Oe represents the treatment with Oenotan; Q represents the treatment with QCTN; V represents the treatments with VR Supra and L represents the pectolytic enzyme Lafase HE Grant Cru. A represents a single dosage of tannin (at AF); B a double dosage of tannin and C a triple dosage of tannin. Three MLF starter cultures Viniflora®oenos (O), CH16(16) and CH35(35) were used to inoculate for MLF in the control and Taniflora treatments, whilst the other treatments were all inoculated only with Viniflora®CH16. The error bars indicate the standard deviation of the repeats.

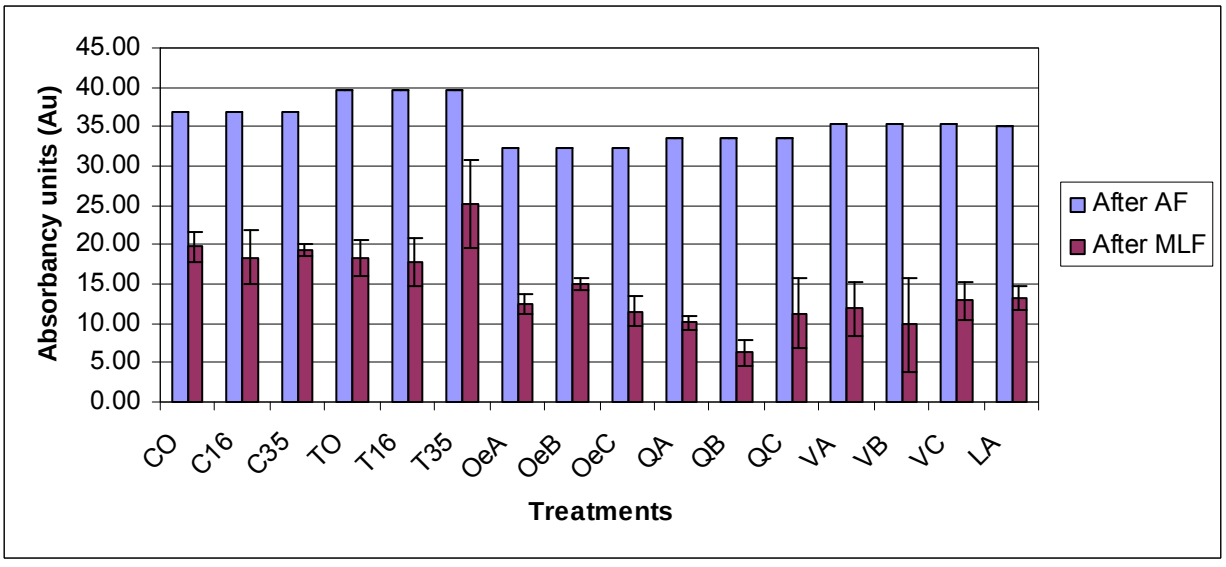


**Figure 5.10** Total red pigments (TRP) after AF and MLF for Merlot 2006. C represents the controls; T represents the treatment with Taniflora; Oe represents Oenotan; Q represents QCTN; V represents VR Supra and L represents Lafase HE Grand Cru. A represents a single dosage of tannin (at AF); B a double dosage of tannin and C a triple dosage of tannin. Three MLF starter cultures Viniflora®oenos (O), CH16(16) and CH35(35) were used to inoculate for MLF in C and T, whilst the other treatments were all inoculated only with Viniflora®CH16. The error bars indicate the standard deviation of the repeats.

#### 5.3.3.3 Total Anthocyanins

The total anthocyanin concentration for the Pinotage wine decreased for all the treatments from approximately 650mg/L to 200 mg/L between AF and MLF (**Figure 5.7**). The percentation decrease was 48% for CO and C35 but 56% for C16. The Taniflora treatments TO and T35 showed a percentation reduction in anthocyanin concentration of 50%, while T16 reduced by 58%. All the other treatments was inoculated with starter culture Viniflora®CH16 and showed reduction percentages that ranged from 61% to 87% in comparison to the 48% (CO, C35) and 50% (TO and T35) of the control and treatments with starter cultures Viniflora®oenos and Viniflora®CH35.

The total anthocyanins were observed to be approximately 100 mg/L less in the Merlot than in the Pinotage wine after AF. For the Merlot wine there was also a decrease from 520 mg/L to 150 mg/L in anthocyanin concentration between AF and MLF. In the treatments with Oenotan and VR Supra tannins the anthocyanins decreased as the level of tannin addition increased. Whilst in the decrease of anthocyanins in the controls showed a possible strain difference. The control with Viniflora®oenos had a lower reduction followed by Viniflora®CH16 and then Viniflora®CH35.

This decrease in anthocyanins could be due to polymerization and precipitation of the anthocyanins (Gonzalez-Nevez *et al*., 2004; Monagas *et al*., 2006). This decrease of anthocyanins was also found in the study by Keulder (2005) up to 6 months of maturation after which it started to stabilise. In all cases the largest decrease was found where starter culture Viniflora®CH16 was used. Vivas *et al*. (1997) showed that LAB can metabolise anthocyanins especially during the growth phase, which could indicate that CH16 have a higher affinity for metabolizing anthocyanins or that the were more cells of CH16 to metabolise it versus the other starter cultures.

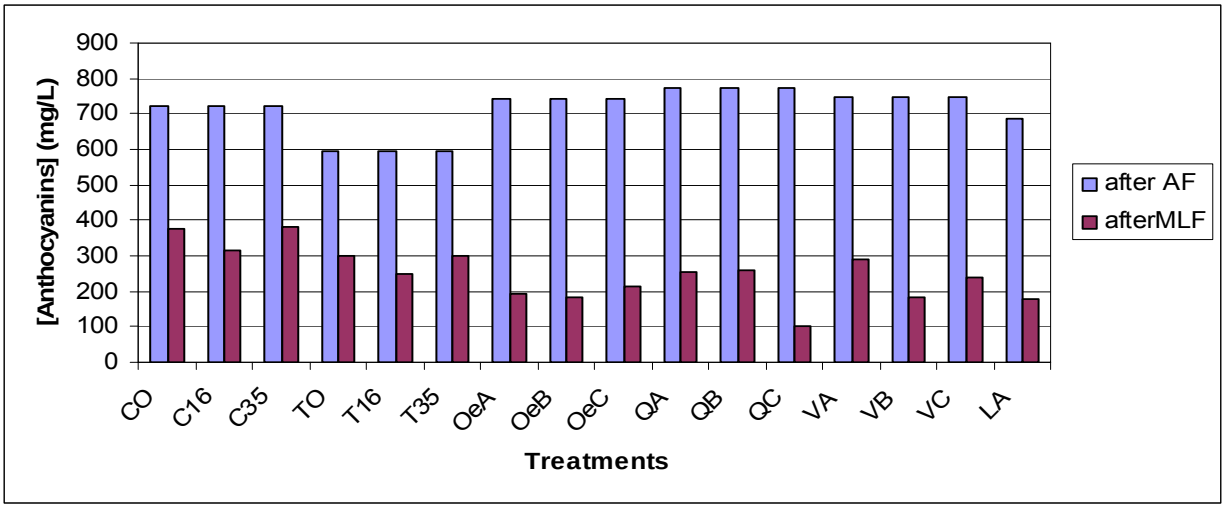


**Figure 5.11** Total anthocyanin concentration after AF and MLF for Pinotage 2006. C represents the controls; T represents the treatment with Taniflora; Oe represents the treatment with Oenotan; Q represents the treatment with QCTN; V represents the treatments with VR Supra and L represents the pectolytic enzyme Lafase HE Grant Cru. A represents a single dosage of tannin (at AF); B a double dosage of tannin and C a triple dosage of tannin. Three MLF starter cultures Viniflora®oenos(O), Viniflora®CH16(16) and Viniflora®CH35(35) were used to inoculate for MLF in the control and Taniflora treatments, while the other treatments were all inoculated only with Viniflora®CH16. Each bar indicates the average of the repeats.

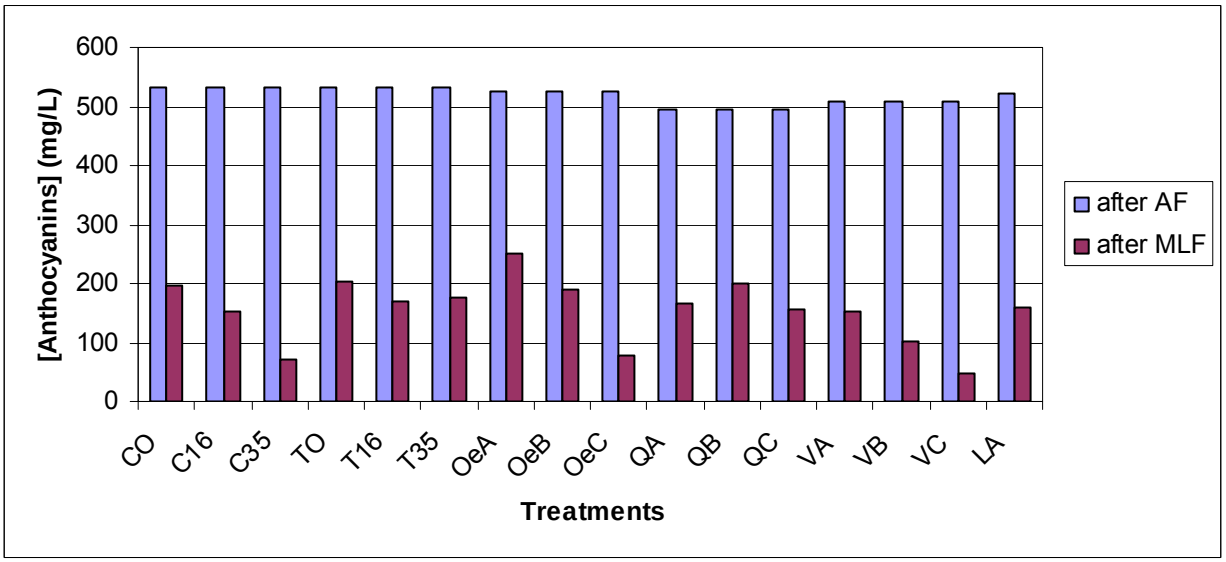


**Figure 5.12** Total anthocyanin concentration after AF and MLF for Merlot 2006. C represents the controls; T represents the treatment with Taniflora; Oe represents the treatment with Oenotan; Q represents the treatment with QCTN; V represents the treatments with VR Supra and L represents the pectolytic enzyme Lafase HE Grant Cru. A represents a single dosage of tannin (at AF); B a double dosage of tannin and C a triple dosage of tannin. Three MLF starter cultures Viniflora®oenos(O), Viniflora®CH16(16) and Viniflora®CH35(35) were used to inoculate for MLF in the control and Taniflora treatments, while the other treatments were all inoculated only with Viniflora®CH16. Each bar indicates the average of the repeats.

### 5.3.3.4 Total phenols

The total phenols decreased after MLF in all the treatments of Pinotage, except for QC and VC which showed a slight increase in phenols (**Figure 5.13**). A decrease in total phenols after MLF was also observed in all the treatments for the Merlot wines, except for treatment QC (**Figure 5.14**). In the Pinotage and Merlot wine the increased dosage of commercial tannins (A, B and C) can be observed especially for QCTN (Q) and VR Supra (V), whilst the triple dosage of Oenotan (OeC) in the Pinotage wine also led to a higher level of total phenols.

The decrease could be as a result of polymerization and the resultant change in extinction coefficients and possible precipitation of these compounds (Mazza *et al*., 1999; Perez-Prieto *et al*., 2003). The addition of pectolytic enzyme (LA) will increase total phenols in wine (Revilla and González-SanJosé, 2003), but this effect will be most prominent during AF and was not observed after MLF. The decrease in total phenols during MLF could also be due to adhesion of the phenolic molecules to bacterial cell walls and should be investigated further.

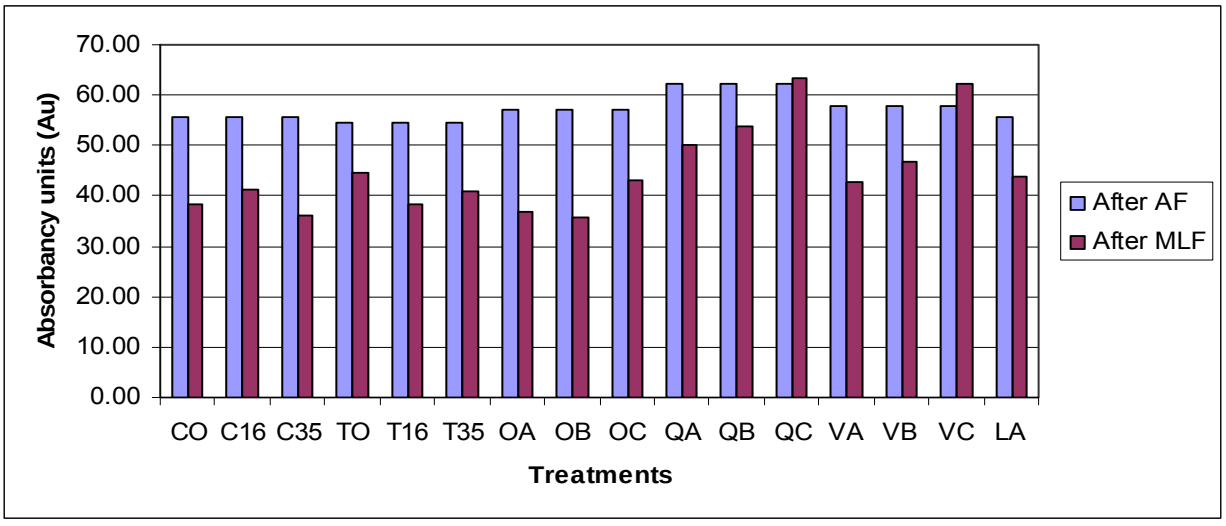


**Figure 5.13** Total phenols after AF and MLF for Pinotage 2006. C represents the controls; T represents the treatment with Taniflora; Oe represents the treatment with Oenotan; Q represents the treatment with QCTN; V represents the treatments with VR Supra and L represents the pectolytic enzyme Lafase HE Grant Cru. A represents a single dosage of tannin (at AF); B a double dosage of tannin and C a triple dosage of tannin. Three MLF starter cultures Viniflora®oenos(O), Viniflora®CH16(16) and Viniflora®CH35(35) was used to inoculate for MLF in the control and Taniflora treatments, whilst the other treatments were all inoculated only with Viniflora®CH16. Each bar indicates the average of the repeats

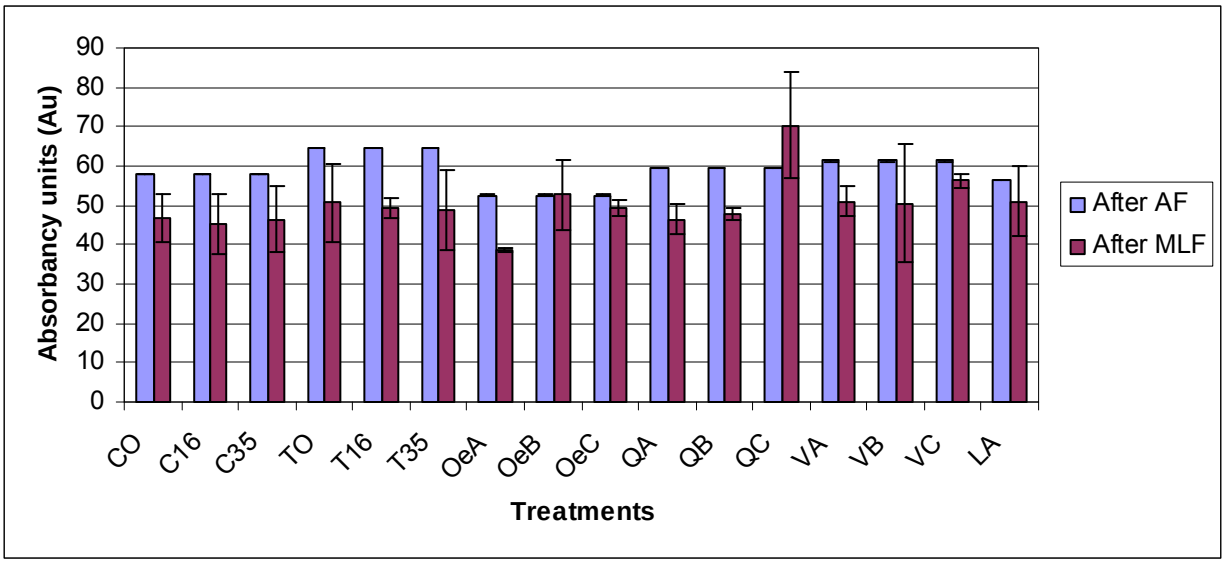


**Figure 5.14** Total phenols after AF and MLF for Merlot 2006. C represents the controls; T represents the treatment with Taniflora; Oe represents the treatment with Oenotan; Q represents the treatment with QCTN; V represents the treatments with VR Supra and L represents the pectolytic enzyme Lafase HE Grant Cru. A represents a single dosage of tannin (at AF); B a double dosage of tannin and C a triple dosage of tannin. Three MLF starter cultures Viniflora®oenos(O), Viniflora®CH16(16) and Viniflora®CH35(35) was used to inoculate for MLF in the control and Taniflora treatments, whilst the other treatments were all inoculated only with Viniflora®CH16. Each bar indicates the average of the repeats and the error bars indicate the standard deviation of the repeats.

## 5.4 CONCLUSION

The commercial tannin additions to the Pinotage and Merlot wines did influence the malic acid degradation during MLF to a certain extent. This influence was observed as a slight inhibition especially during the first week for the Pinotage wine in the case of all the commercial tannins. In the Merlot wine a slight stimulatory effect of the tannin VR Supra could be observed on the degradation of malic acid, while no effects was visible for the other treatments. In most cases the largest difference was found between the triple dosage of tannin and the control. Therefore if commercial tannins are added to wine as prescribed by the suppliers no or little effect will be observed on MLF, since MLF finished at the same time for all the treatments. The full constitution of these commercial tannin products used during the study is not known and it is effect on MLF must herefore be further investigated. The biogenic amine data, revealed strain differences in the amount of BA's produced as well as differences between BA levels and tannin dosage. The phenolic compounds developed as expected accept in the case of the CD of the Pinotage which was influenced by the decrease of the pH with tartaric acid during MLF.

Further work could entail monitoring of the development of the MLF starter cultures through enumeration on a weekly basis during MLF in an experimental layout similar to this one. The pH increase of the wine during MLF could be monitored in comparison with the decrease in CD on a weekly basis, to observe the extent of CD loss through MLF. Wine could also be inoculated for MLF with LAB with β-Glucosidase activity in combination with condensed tannins, to assess it's effect on anthocyanin esterified with glucose, which could be utilised by such bacteria.

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# 6. RESEARCH RESULTS

# Assessing different inoculation times of malolactic fermentation starter cultures in high pH red wines

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## ABSTRACT

Spontaneous malolactic fermentation (MLF) during alcoholic fermentation (AF) often causes sluggish AF. Early inoculation with selected *Oenococcus oeni* strains may reduce this risk, suppressing wild bacteria, and at the same time conducting a more controlled MLF without influencing the wine aroma or hazardous compounds (biogenic amines (BA's)) for human health negatively. This is especially important in red wine with a high pH, where spontaneous MLF occurs frequently, already during AF, causing stuck AF and rise in volatile acidity (VA). Two different MLF starter cultures, Enoferm alpha and Lalvin VP41, were inoculated in combination with two different yeast cultures, Lalvin ICDV-254 and Lalvin L2056. The inoculation times were (A) simultaneous inoculation of the bacteria and yeast, (B) inoculation of the bacteria at 30 g/L residual sugar and (C) inoculation right after AF. Balling readings, cell counts, and routine wine analysis were done as well as the measurement of the amount of BA's after MLF. The controls were inoculated for AF but MLF was left to occur spontaneously. AF as well as MLF were finished within 21 days for all the treatment A's, whilst treatment B and C finished MLF 2 weeks later and showed more or less the same degradation rate. The BA levels were also lower for these treatments versus the other inoculations B and C.

## 6.1 INTRODUCTION

Malolactic fermentation (MLF) starter cultures are inoculated into wine to initiate MLF and in most cases these starter cultures consist of pure culture of *Oenococcus oeni* isolated from wine. As in the case of yeast starter cultures for alcoholic fermentation (AF), the use of MLF starter cultures enables the winemaker to have more control over the fermentation and the outcome thereof. An important factor of controlling AF and MLF is the timely initiation of the process.

When MLF fails to start it could be very costly, since it would influence the aroma of wine and therefore wine quality. After AF the wine is not protected by adequate levels of $SO_2$ anymore and therefore, when LAB (spontaneous or starter cultures) fail to successfully induce MLF, the consequence could be infection by other microorganisms like spoilage strains of LAB, acetic acid bacteria (AAB) and *Brettanomyces* or *Dekkera* yeasts. Spoilage aromas of *Brettanomyces/Dekkera* yeasts, refer to compounds like 2-ethyltetrahydopyridine, 2-acetethyltetrahydopyridine and 2-acetylpyroline, which manifest as a mousy off-flavour in wine (Snowdown *et al*., 2006). LAB off-flavours such as boilt cabbage, cauliflower, rancid and buttery flavours could develop from amino acid catabolism (Ardö, 2006), as well as high levels of acetic acid, acetaldehyde, acetoin and diacetyl can develop (Fornachon and Lloyd, 1965; Henick-Kling *et al*., 1994; Kandler, 1983; Mascarenhas, 1984). AAB are known to convert ethanol to acetic acid in the presence of small concentration of oxygen (Greenshields, 1978; Drysdale and Fleet, 1989).

MLF starter cultures are usually inoculated either at the beginning of AF with the yeast or after AF (Henick-Kling, 1993). Starter culture failures are mostly the result of not implementing the proposed inoculation procedures as prescribed, but can sometimes also fail because of antagonistic interactions between yeasts and bacteria. These antagonistic interactions can be due to ethanol production, $SO_2$ and the competition for nutrients that may inhibit bacterial growth (Bisson and Kunkee, 1991; Lonvaud-Funel *et al*., 1988a; Lonvaud-Funel *et al*., 1988b). The interactions between wine yeasts and malolactic bacteria are recognised as having a potential impact on bacterial growth and MLF activity (Fornachon, 1968; King and Beelman, 1986; Henick-Kling and Park, 1994; Nygaard and Praal, 1996).

During MLF biogenic amines (BA's) are also formed by LAB. It has been reported by various authors that the levels of BA's increase after MLF (Granchi *et al*., 2005;

Lonvaud-Funel, 1999, 2001; Marcobal *et al*., 2006; Ribéreau-Gayon *et al*., 2000; Soufleros *et al*., 1998). Histamine, tyramine, putrescine and cadaverine are the major BA's found in wine (Lonvaud-Funel, 2001). The formation of these BA's has been associated with a lack of hygiene during the winemaking process.

The aim of this study is to evaluate different inoculation times of yeasts and bacteria in high pH red wines to possibly reduce the risks of spoilage due to spontaneous MLF.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Experimental layout

Cabernet Sauvignon grapes from South Africa (Stellenbosch region) with a high pH (3.9) was used for this experiment. The wine was inoculated with two different yeast strains in combination with two different MLF starter cultures and three different inoculation strategies, namely (A) Simultaneous inoculation of bacteria and yeasts at the beginning of AF; (B) inoculation of MLF starter culture when 30 g/L residual sugar still remaining in the must; (C) inoculation after AF have been completed (RS < 3 g/L) (**Table 6.1**). Two different yeast strains, Lalvin ICV-D254 (254) and Lalvin L2056 (2056) (Lallemand, South Africa) and two MLF starter cultures, Enoferm alpha (α) and Lalvin VP41 (41) (Lallemand, South Africa), was used for this experiment. The inoculation rates as prescribed on the packaging were used.

**Table 6.1 Sample layout and codes that will be used throughout the study**

| | **no** | **Enoferm alpha** | **no** | **Lalvin VP41** | **no** | **Enoferm alpha** |
|---|---|---|---|---|---|---|
| ICDV | 1 | 254αA1*** | 3 | 254.41.A1 | 9 | 254αB1 |
| 254* | 2 | 254αA2 | 4 | 254.41.A2 | 10 | 254αB2 |
| Lalvin | 5 | 2056αA1 | 7 | 2056.41.A1 | 13 | 2056αB1 |
| 2056** | 6 | 2056αA2 | 8 | 2056.41.A2 | 14 | 2056αB2 |
| | **no** | **Lalvin VP41** | **no** | **Enoferm alpha** | **no** | **Lalvin VP41** |
| ICDV | 11 | 254.41.B1 | 17 | 254αC1 | 19 | 254.41.C1 |
| 254* | 12 | 254.41.B2 | 18 | 254αC2 | 20 | 254.41.C2 |
| Lalvin | 15 | 2056.41.B1 | 21 | 2056αC1 | 23 | 2056.41.C1 |
| 2056** | 16 | 2056.41.B2 | 22 | 2056αC2 | 24 | 2056.41.C2 |

* controls for ICDV 254 are duplicates number 25 and 26

** controls for Lalvin 2056 are duplicates number 27 and 28

*** numbers 1 and 2 refers to the duplicate assigned

### 6.2.2 Winemaking procedures

The Cabernet Sauvignon grapes were harvested at a very high sugar level of 28 ˚B. If taken into account that the general conversion rate of sugar to ethanol by yeast is roughly 55%, that will lead to an alcohol content of 15.6%. If MLF were to be conducted after AF on this wine it would be extremely difficult, since the MLF starter cultures might be inhibited by these high alcohol levels. Therefore the juice was diluted by removing 10 L of juice and replacing it with 10 L of distilled water. The juice was then 22.3 ˚B. The pH and titratable acidity (TA) were only measured after the dilution and the TA then adjusted accordingly to 6.5 g/L.

The Cabernet Sauvignon was crushed and 30 ppm $SO_2$ was added. The must was homogenised and then divided into 28 10 L buckets with lids. Each sample bucket consisted of 3.8 kg skins and 1.2 L free running juice. The buckets were inoculated with the two different yeasts, which resulted into 14 buckets fermenting with Lalvin ICV-D254 and 14 with Lalvin L2056. Go-Ferm (Lallemand, South Africa) was used to re-hydrate the yeast in and Fermaid K (Lallemand, South Africa) was added at mid fermentation as a nutrient supplementation to the fermentation.

The inoculation of the MLF starter cultures were then performed as described under 6.2.1. Controls (254C and 2056C) for this experiment were inoculated for AF (Lalvin ICV-D254 and Lalvin L2056) and then left to undergo a spontaneous MLF. After AF when the residual level were < 5˚B the sample buckets were pressed separately and the wine was homogenised and then divided into duplicate samples of 2 L glass containers which were closed with rubber stoppers containing a S-shaped airlocks.

### 6.2.3 Yeast and bacterial strains and culture conditions

MRS agar (Biolab, Merck, South Africa) enriched with 20% apple juice (containing no preservatives) (MRSA) (pH5.2). The addition of apple juice and lowering of the pH stimulates the growth of *O. oeni*. Normal De Man, Rogosa and Sharpe (MRS) agar was also used for the enumeration of LAB. Both MRSA and MRS contained 100 mg/L Actistab (50% glucose, 50% natamycin, Gist-brocades, France, S.A.), dissolved in methanol, for the inhibition of moulds, fungi and yeast. Kanamycin sulphate ($C_{18}H_{36}N_4O_{11}$ X $H_2SO_4$,

Roche Diagnostics, dissolved in sterile distilled water) at 25 mg/L, were used for the inhibition of AAB.

Yeast Peptone Dextrose (YPD) agar (Merck, Biolab Diagnostics (Pty) Ltd) was used for the enumeration of yeast. Kanamycin sulphate was also used in the YPD media for the inhibition of AAB and 50 mg/L Nisin (Sigma-Aldrich Co., dissolved in methanol) for the inhibition of LAB.

GYC agar plates (5% glucose, 10% yeast extract, 3% $CaCO_3$, 2% agar) were used for the enumeration of AAB. Actistab and Nisin were used to inhibit yeast and LAB respectively.

The MRSA and MRS was incubated under facultative anaerobic conditions at 30˚C (Anerogen, Oxoid) in an anaerobic jar (Davies Diagnostics (Pty) Ltd.), whilst YPD and GYC media was incubated at 30˚C aerobically.

Test tubes filled with 9 mL distilled water, which was then autoclaved, was used to make dilution series from $10^{-1}$-$10^{-6}$.

### 6.2.4 Analyses of wine

AF was monitored by measuring the sugar level of the must by using a hydrometer. L-Malic acid was measured weekly from inoculation of the starter culture using Fourier Transformation Infrared Spectroscopy (FT-IR) (Foss Grape scan). Samples were filtered with a Filtration Unit (type 79500, FOSS Electric, Denmark) connected to a vacuum pump. The filter unit uses filter paper circles graded at 20 – 25 µm with diameter 185 mm (Schleicher & Schnell, reference number 10312714). The filtered must were used for FT-IR spectral measurements. A Winescan FT120 equipped with a purpose built Michelson interferometer was used to generate the FT-IR spectra (FOSS Electric A/S, Hillerød, Denmark). Instrument settings included: cell path length of 37 µm, sample temperature set to 40°C, and sample volume of 7 – 8 ml. The sample is pumped through the heat exchanger and the $CaF_2$-lined cuvette. Samples are scanned from 5011 to 926 $cm^{-1}$ at 4 $cm^{-1}$ interval.

Other analysis that was done with FT-IR spectroscopy was the measurement of routine wine parameters (pH, Total acidity (TA), Volatile acidity (VA), Malic acid, Lactic acid, Ethanol, Glucose, Fructose and Glycerol) at different stages of the winemaking process. For instance the juice after crushing and the wine after AF were analysed for these above mentioned parameters.

After completion of MLF samples were sent to Distell, South Africa to analyse the levels of biogenic amines by using high-pressure liquid chromatography (HPLC) (Alberto *et al*., 2002).

## 6.3 RESULTS AND DISCUSSION

### 6.3.1 Rate of alcoholic and malolactic fermentation

Alcoholic fermentation developed the same in all the treatments (**Figures 6.1** and **6.2**). This indicates that the inoculation time of different bacteria do not affect the yeast starter cultures, even if inoculated simultaneously. In similar studies by Grossmann *et al*. (2002), Henick-Kling and Park (1994), Jussier *et al*. (2006) and Rauhut *et al*. (2001) it was also found that the addition of MLF starter cultures together with the yeast starter cultures did not inhibit the yeast and therefore the alcoholic fermentation.

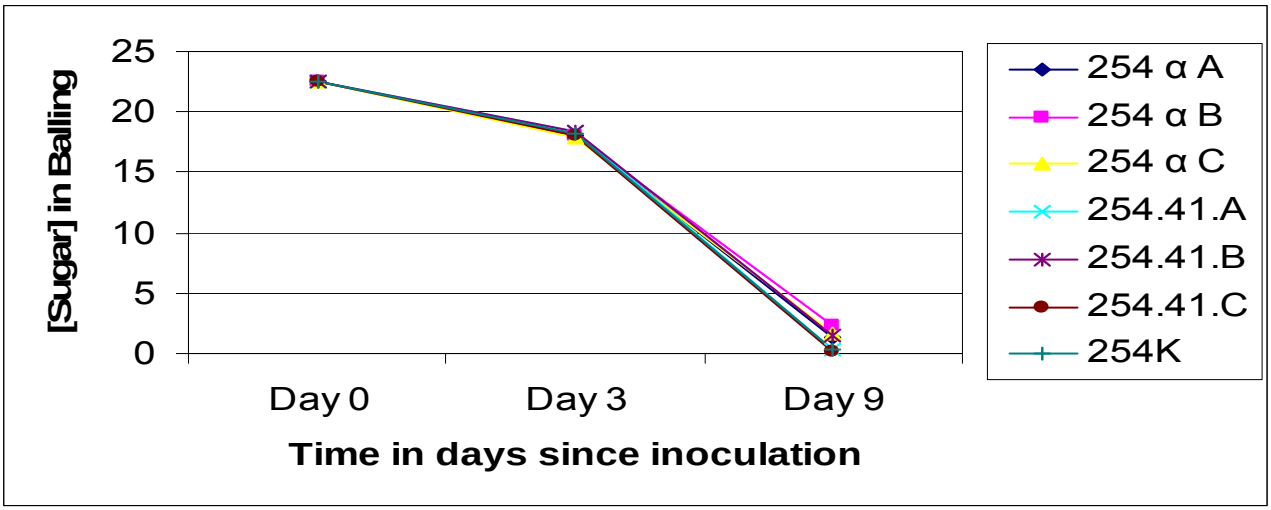


**Figure 6.1** Alcoholic fermentation. In all the samples ICV D254 (254) were used to inoculate AF and Enoferm alpha (α) and Lalvin VP41 (41) was used to conduct MLF, except for the control sample which was a spontaneous MLF. 254C represents the control; A simultaneous inoculation of AF and MLF starter cultures; B Inoculation of MLF starter culture at < 30 g/hL RS; C inoculation of MLF starter culture at the end of AF.

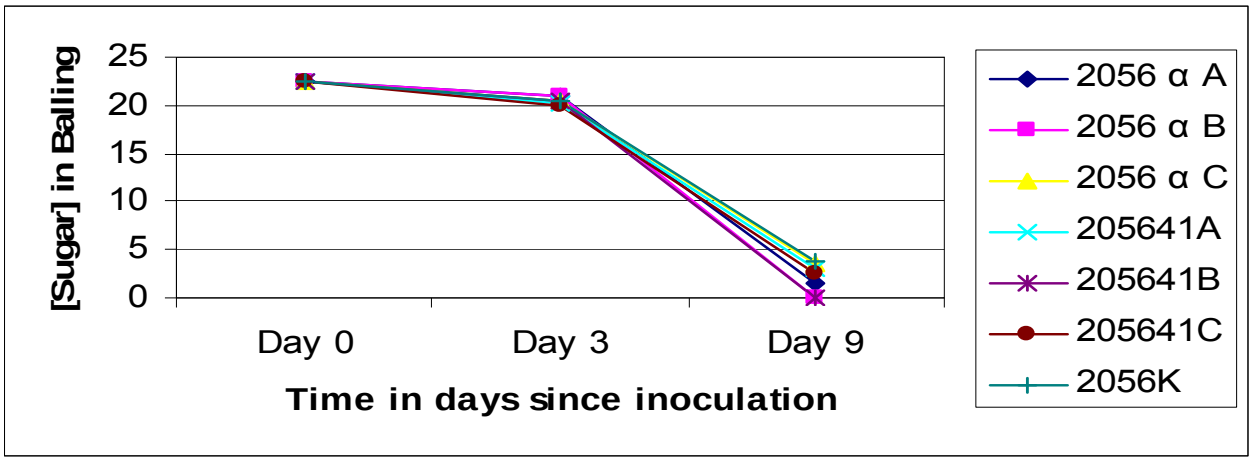


**Figure 6.2** Alcoholic fermentation. In all the samples Lalvin 2056 (2056) were used to inoculate AF and Enoferm alpha (α) and Lalvin VP41 (41) was used to conduct MLF, except for the control sample which was a spontaneous MLF. 2056C represents the control; A simultaneous inoculation of AF and MLF starter cultures; B Inoculation of MLF starter culture at < 30 g/hL RS; C inoculation of MLF starter culture at the end of AF.

The malic acid degradation was monitored in the samples from inoculation of the yeast starter cultures until the end of MLF (< 0.3 g/L malic acid). After week 1 of MLF the two MLF starter cultures (Enoferm alpha, Lalvin VP41) that were inoculated in combination with ICV D254, showed the highest reduction in malic acid (**Figures 6.3** and **6.4**). The reduction was 64% and 76% respectively for 254αA and 254.41.A, whilst the other treatments (254C, 254αB, C and 254.41.B, C) varied between 30%-38%. This higher level of reduction for the A treatments continued through weeks 2 (81%) and 3 (89%) and no malic acid was detected from week 4 onwards. The same results were found in the case where the two MLF starter cultures (Enoferm alpha and Lalvin VP41) were inoculated in combination with Lalvin 2056 yeast (**Figures 6.5** and **6.6**). The percentage of malic acid reduction over the first 3 weeks of MLF was as follows for 2056αA and 2056.41.A respectively: 66% and 77% week1; 81% and 82% week 2 and 90% and 93% in week 3. No malic acid was detected for these treatments from week 4 to the end of the sampling period (week 6). Therefore the treatments A for this study finished AF and MLF within 21 days of inoculation. In a similar experiment by Henick-Kling and Park (1994) done in grape juice medium, simultaneous inoculation of yeast and bacterial starter cultures were able to conduct and finish MLF within 9-20 days. Grossmann *et al*. (2002), found that AF and MLF was finished in Riesling wine within 14 days since preparation of the must.

The results also correlate with cell numbers obtained at the end of MLF, since the treatment A's had higher cell numbers than the other treatments (254C, 254αB, C and 254.41.B, C) at this stage (**Figure 6.7**).

The control and other 2 treatments (B and C) for the inoculation of Enoferm alpha and Lalvin VP41 in combination with yeast ICV D254 (**Figures 6.3** and **6.4**), the pattern for malic acid degradation rate over week 2 and 3 were similar, with B>C>Control. Over weeks 4 and 5 the highest malic acid degradation rate shifted between treatments B and C, while the control samples remained at the lowest malic acid degradation rate. In week 6 no malic acid was detected for the control sample (spontaneous MLF), while treatments B and C were at a reduction level of 88% and 91% for Enoferm alpha and 86% (B and C) for LalvinVP41 (technically finished with MLF, since malic acid between 0.27 and 0.42 g/L). Gallander (1979) also found that inoculation during or after MLF led to approximately the same time for completing MLF.

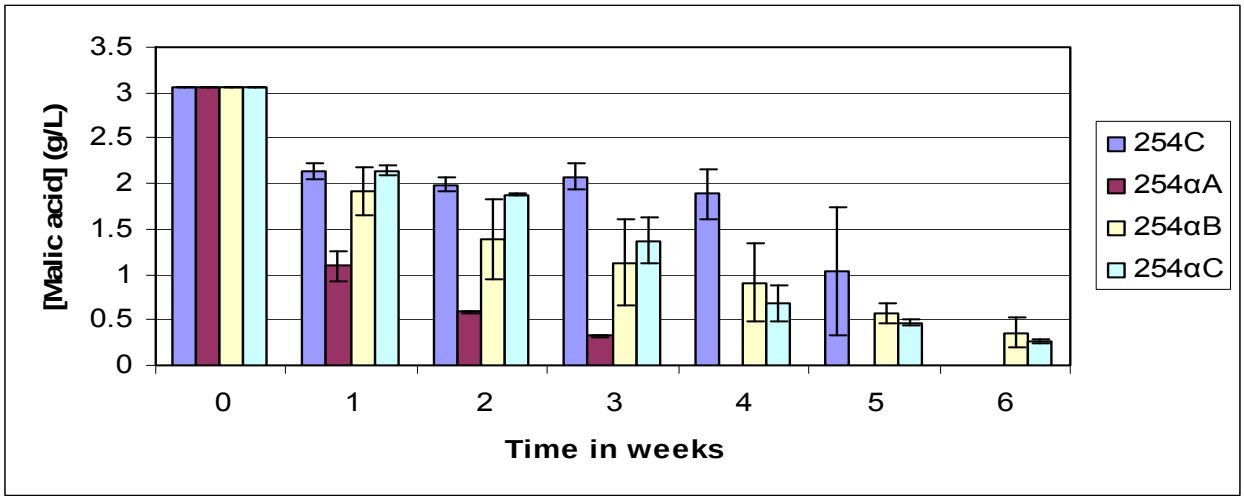


**Figure 6.3** The degradation of malic acid from inoculation until the end of malolactic fermentation. In all the samples ICV D254 (254) were used to inoculate AF and Enoferm alpha (α) was used to conduct MLF, except for the control sample which was a spontaneous MLF. 254C are the control; A simultaneous inoculation of AF and MLF starter cultures; B Inoculation of MLF starter culture at < 30 g/hL RS; C inoculation of MLF starter culture at the end of MLF. No malic acid was detected for 254αA during weeks 4, 5 and for 254C in week 6. The error bars indicate the standard deviation of the duplicates.

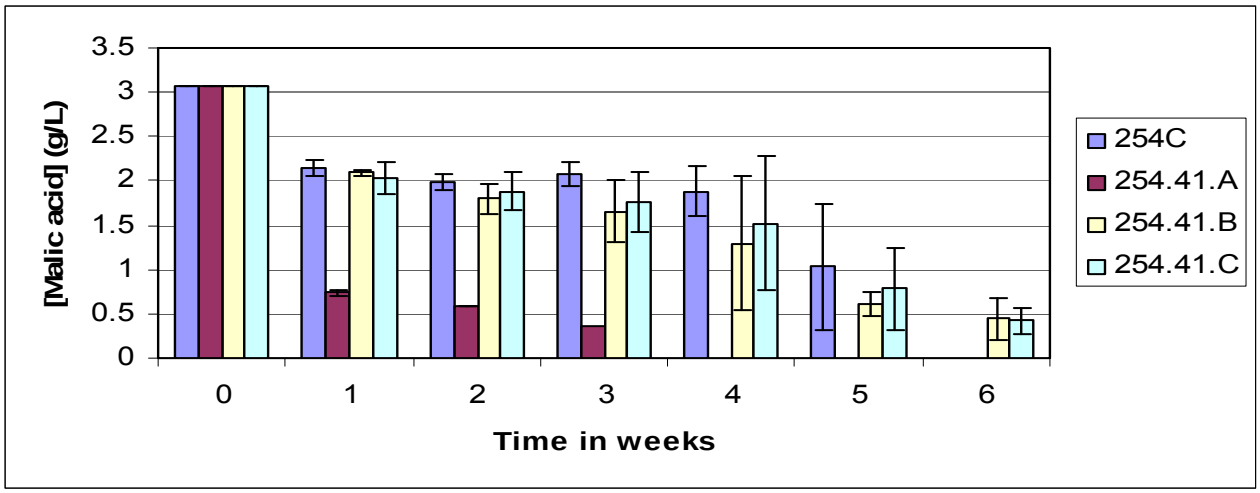


**Figure 6.4** The degradation of malic acid from inoculation until the end of malolactic fermentation. In all the samples ICV D254 (254) were used to inoculate AF and Lalvin VP41 (41) was used to conduct MLF, except for the control sample which was a spontaneous MLF. 254C are the control; A simultaneous inoculation of AF and MLF starter cultures; B Inoculation of MLF starter culture at < 30 g/hL RS; C inoculation of MLF starter culture at the end of MLF. No malic acid was detected for 254.41.A during weeks 4, 5 and for 254C in week 6. The error bars indicate the standard deviation of the duplicates.

During week 2 and 3 of MLF for the samples where Enoferm alpha and VP 41 was inoculated with yeast Lalvin L2056 (**Figure 6.5** and **6.6**) the control and treatment B samples had a higher rate of malic acid degradation than treatment C. These samples (Control, Treatment B and C) reached more or less the same level of malic acid degradation at week 4 of MLF. This pattern continued over weeks 5 and 6 with the exception of treatment B that was not detected at week 6. During this inoculation combination of the two MLF starter cultures (Enoferm alpha and Lalvin VP41) with Lalvin 2056 yeast the treatment B's finished MLF within 5 weeks. The control which was inoculated AF with spontaneous MLF (2056C) got stuck at a level of 72% reduction of malic acid.

The cell numbers at the end of MLF for treatments B and C where yeast Lalvin L2056 was inoculated with Enoferm alpha MLF starter culture (2056αB, C), is much lower than the treatment A (Simultaneous inoculation, 2056αA) (**Figure 6.7**). This could be because of inhibitory effects due to the presence of the higher ethanol concentration in the wine at the stage when treatments B and C were inoculated.

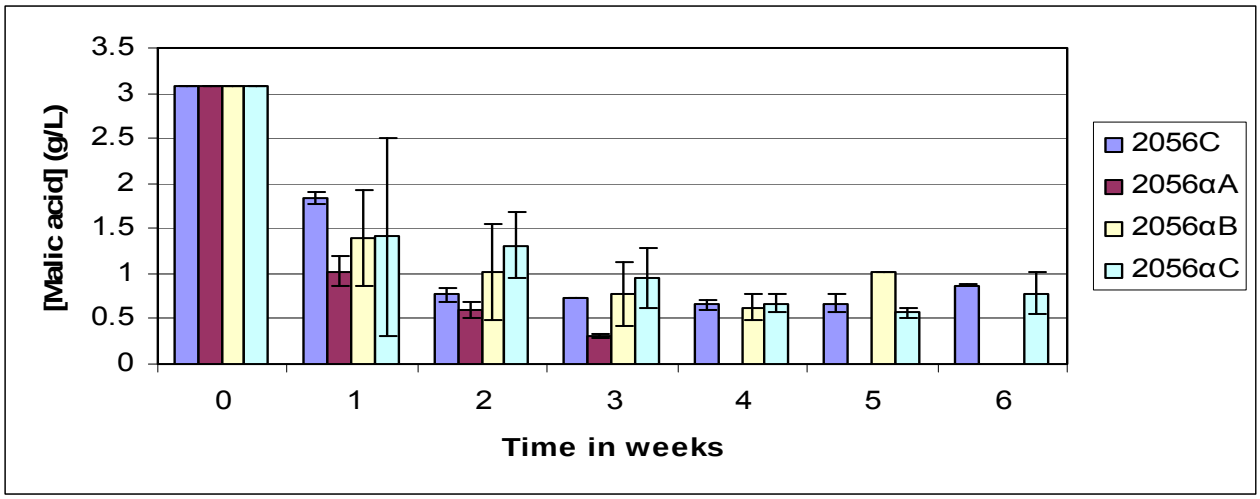


**Figure 6.5** The degradation of malic acid from inoculation until the end of malolactic fermentation. In all the samples Lalvin 2056 (2056) were used to inoculate AF and Enoferm alpha (α) was used to conduct MLF, except for the control sample which was a spontaneous MLF. 2056C are the control; A simultaneous inoculation of AF and MLF starter cultures; B Inoculation of MLF starter culture at < 30 g/hL RS; C inoculation of MLF starter culture at the end of MLF. No malic acid was detected for 2056αA during weeks 4, 5 and for 2056αB in week 6. The error bars indicate the standard deviation of the duplicates.

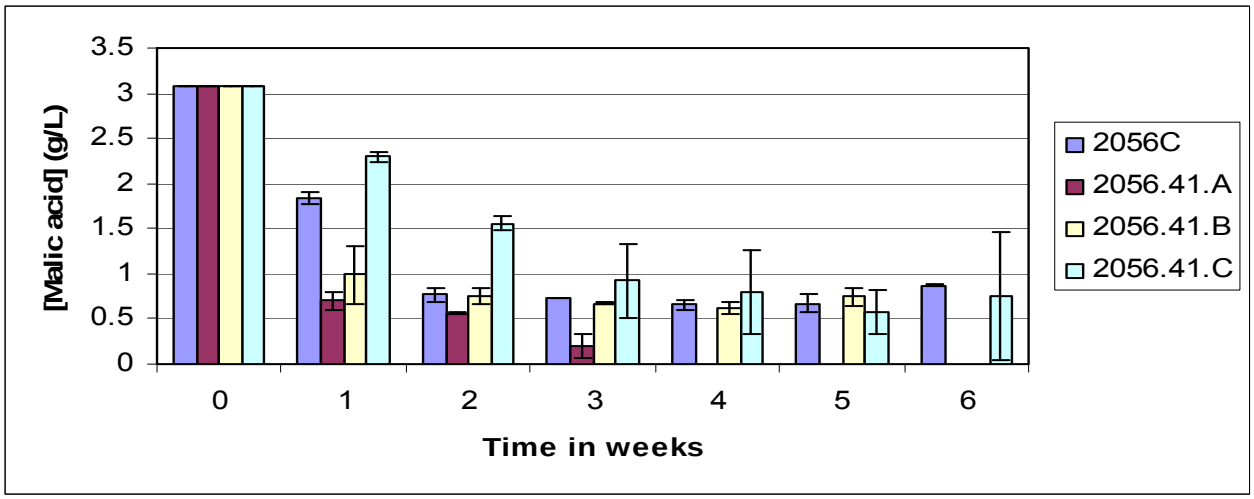


**Figure 6.6** The degradation of malic acid from inoculation until the end of malolactic fermentation. In all the samples Lalvin 2056 (2056) were used to inoculate AF and Lalvin VP41 (41) was used to conduct MLF, except for the control sample which was a spontaneous MLF. 2056C are the control; A simultaneous inoculation of AF and MLF starter cultures; B Inoculation of MLF starter culture at < 30 g/hL RS; C inoculation of MLF starter culture at the end of MLF. No malic acid was detected for 2056.41.A during weeks 4, 5 and 6 for 2056.41.B in week 6. The error bars indicate the standard deviation of the duplicates.

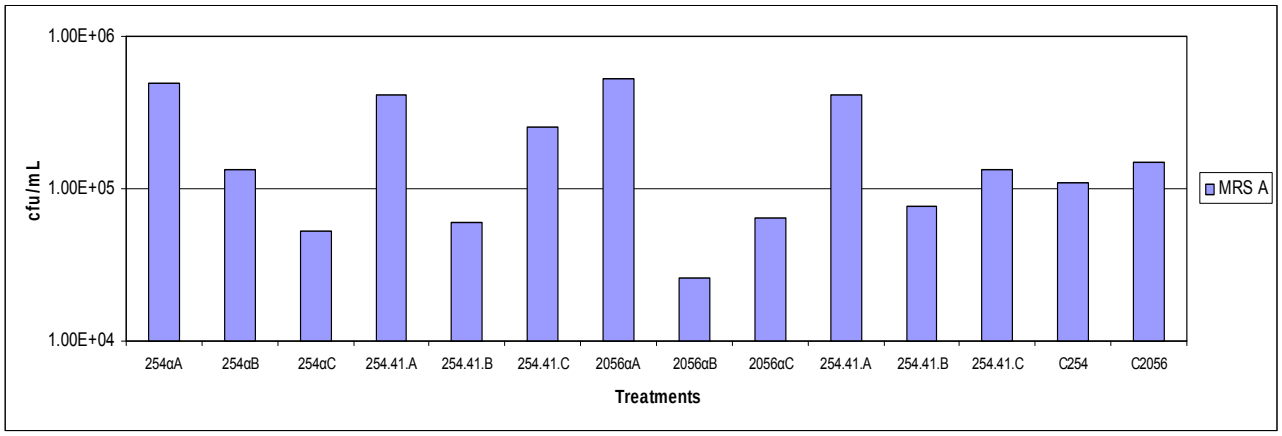


**Figure 6.7** Cell concentrations at the end of malolactic fermentation for the controls and treatments. 254 represents ICD V254 yeast starter; 2056 represents the Lalvin 2056 yeast starter; α represents Enoferm alpha MLF starter culture; 41 represents the Lalvin VP41 MLF starter culture; C are the control; A simultaneous inoculation of AF and MLF starter cultures; B Inoculation of MLF starter culture at < 30 g/hL RS; C inoculation of MLF starter culture at the end of MLF. Each bar indicates the average of the cell concentrations of the duplicates.

### 6.3.2 Biogenic amines

In wine the level of biogenic amines (BA's) that will develop, depends on various factors. For instance grape variety and viticultural practices (Soufleros *et al*., 1998), yeast metabolism and LAB. LAB differs in their capability to produce BA's on a strain level and the factor that mostly influences this capability is pH. A high pH sometimes yields a higher BA level in wine (Lonvaud-Funel, 2001). In this experiment a high pH (3.9) Cabernet Sauvignon was used and therefore BA's could potentially occur at a higher level than at normal wine pH (3.4-3.6). Enoferm alpha and Lalvin VP41 are both described by Lallemand to produce very low levels of biogenic amines. The biogenic amine levels were measured after MLF for all the treatments (**Table 6.2).**

All the simultaneous inoculated combinations resulted in no histamine and tyramine production (254α,41A; 2056α, 41A) (**Figures 6.8** and **6.9**). Histamine have been found to be present before alcoholic fermentation in grape must (Bertoldi *et al*., 2004; Vidal Carou *et al*., 1990), but in a study by Marcobal *et al*. (2006) no histamine or tyramine was detected in the must even though it was rich in the precursor amino-acid histadine. Therefore their results suggested that these amines are mostly produced by biological decarboxylation during fermentation. The fact that the simultaneous inoculations did not develop histamine or tyramine could be very important for winemaking, since these two amines are the most toxic amines found in wine (Stratton *et al*., 1991). Legislation has not yet been established with regards to these amines but upper limits have been suggested for histamine in various countries.

**Table 6.2** Biogenic amine levels in mg/L measured at the end of MLF for all the treatments. Each level indicated the average of the duplicates.

| Sample ID | Histamine | Tyramine | Putrescine | Cadaverine |
|---|---|---|---|---|
| 254αA | nd* | nd | 3.22 | 0.64 |
| 254αB | 2.06 | 1.48 | 11.62 | 0.91 |
| 254αC | 2.85 | 2.20 | 20.88 | 1.44 |
| 254.41.A | nd | nd | 3.53 | 0.85 |
| 254.41.B | 10.03 | 5.52 | 13.47 | 1.36 |
| 254.41.C | 9.47 | 5.42 | 9.54 | 1.00 |
| 2056αA | nd | nd | 2.54 | 0.63 |
| 2056αB | nd | nd | 6.55 | 0.32 |
| 2056αC | nd | 1.54 | 9.06 | 0.57 |
| 2056.41.A | nd | nd | 2.05 | 0.54 |
| 2056.41.B | 5.94 | 3.75 | 13.08 | 1.84 |
| 2056.41.C | 6.33 | 2.71 | 10.96 | 1.14 |
| 254C | 0.87 | 2.81 | 11.12 | 1.47 |
| 2056C | nd | 2.13 | 8.32 | 0.63 |

*not detected

These upper limits are in mg/L: Germany, 2; Holland, 3; Finland, 5; Belgium, 5-6; France, 8; Switzerland and Austria, 10 (Busto *et al*., 1996; Lehtonen, 1996). When looking at the remaining treatments, the combination of Lalvin ICV-D254 with Enoferm alpha of inoculation treatments B and C (254αB, C) and the combination of Lalvin ICV-D254 with Lalvin VP41 starter culture of inoculation treatments B and C (254.41.B, C) developed a histamine level of 2-3 mg/L and 10mg/L respectively (**Figure 6.8**). These results show that strain differences exist with regards to the amount of amines that were produced, which have also previously been reported by other authors (Guerrini *et al*., 2002; Moreno-Arribas *et al*., 2003). In the case of the combination of yeast Lalvin L2056 with the two starter cultures for inoculation treatments B and C it was found that the combination with starter culture Enoferm alpha (2056αB, C) had no histamine or tyramine production in 2056αB as well as no histamine, but a low level of tyramine produced in 2056αC (1.54 mg/L) (**Figure 6.9**). When Lalvin L2056 was inoculated with MLF starter culture Lalvin VP41 for inoculation treatments B and C (2056.41.B, C) histamine and tyramine was produced at 6 mg/L and 3mg/L respectively. This is possibly due to the natural LAB present in the fermentation that had a longer time to play a role before the inoculated MLF starter culture

to dominate. This is also shown in the spontaneous MLF where histamine and tyramine, was produced by the same natural LAB.

The control that underwent AF with Lalvin ICV-D254 (254C) produced histamine (0.87 mg/L) and tyramine (2.81 mg/L) (**Figure 6.8**) while the control that underwent AF with yeast Lalvin L2056 did not show histamine after MLF but tyramine was detected at 2.13 mg/L (**Figure 6.9**). These amines in the controls were most likely formed by the natural occurring LAB, since spontaneous MLF occurred. It also showed that co-inoculation takes prevalence over spontaneous MLF since no histamine or tyramine was detected in the co-inoculation treatments (A).

The other biogenic amines that were measured after MLF were putrescine and cadaverine. These amines on their own are not very toxic but it potentiates the toxic effects of histamine and tyramine (Stratton *et al*., 1991). Putrescine was the biogenic amine that was present at the highest levels of all the amines measured (2-20 mg/L). Marcobal *et al*. (2006) also found that putrescine was the most abundant in must and therefore must be mainly associated with grape variety as suggested by previous data (Bertoldi *et al*., 2004), and after vinification it was again the most abundant in the wine. Where MLF was performed with Enoferm alpha starter culture in combination with both yeast starter cultures (Lalvin ICV-D254 and Lalvin L2056) the levels of putrescine produced showed the following order: 254αC>254αB>254αA (21, 12 and 3 mg/L) (**Figure 6.8**) and 2056αC>2056αB>2056αA (9, 7 and 3 mg/L) (**Figure 6.9**). In the case where MLF was performed with Lalvin VP41 the pattern of putrescine levels had the following order: 254.41.B>254.41.C>254.41.A (13, 10 and 4 mg/L) (**Figure 6.8**) and 2056.41.B>2056.41.C>2056.41.A (13, 11 and 2 mg/L) (**Figure 6.9**). Again in all instances the lowest amounts of putrescine was produced by the co-inoculation treatments. The controls (254C and 2056C) had putrescine levels of 11 and 8 mg/L respectively which more or less correlated with the treatments C. Cadaverine was present in all the treatments and controls but at very low levels. It also seems that Enoferm alpha has a shorter lag phase and therefore suppresses the activity of the natural LAB faster. This can be seen as Enoferm alpha in treatments B and C has lower biogenic amine levels in general.

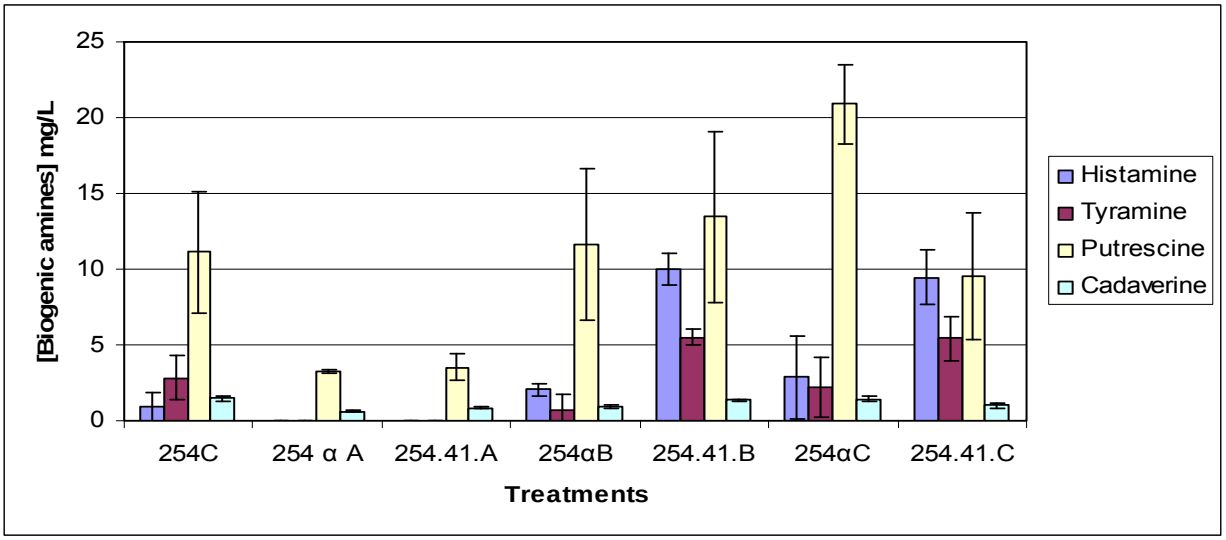


**Figure 6.8** Biogenic amine levels for treatment A, B and C after MLF. 254C represent the control sample that was fermented with Lalvin ICV-D254 but which underwent spontaneous MLF. A represents simultaneous inoculation of yeast and bacteria; B represents inoculation at <30 g/L RS; C represents inoculation after AF; 254 Lalvin ICV-D254 yeast used for AF; α represents MLF starter culture Enoferm alpha; 41 represents Lalvin VP41 MLF starter culture. No histamine and tyramine was detected for the treatments A, as well as no histamine in control 2056. Each bar indicates the average of the replicates.

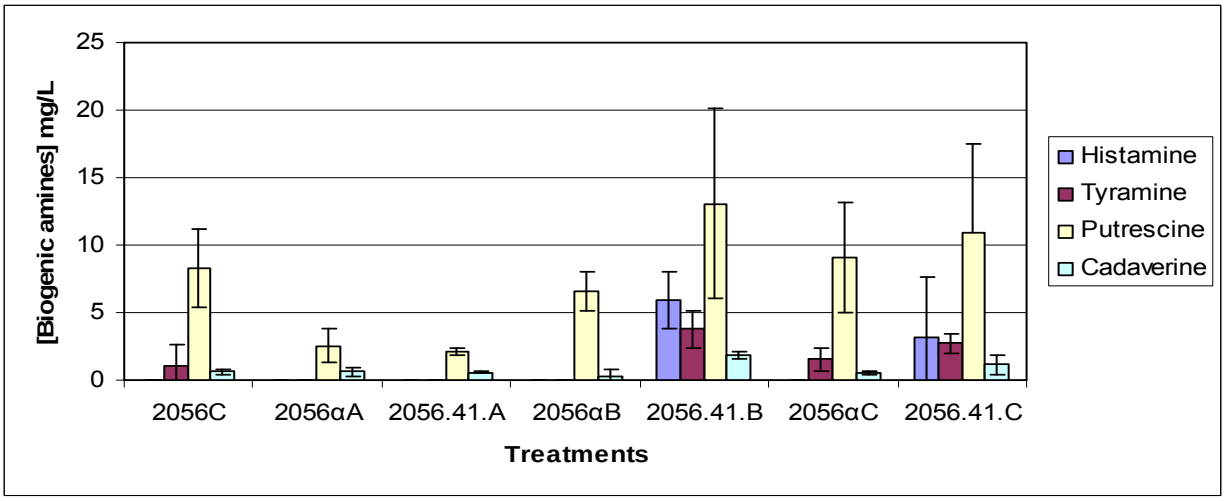


**Figure 6.9** Biogenic amine levels for treatment A, B and C after MLF. 2056C represent the control sample that was fermented with Lalvin L2056 but which underwent spontaneous MLF. A represents simultaneous inoculation of yeast and bacteria; B represents inoculation at <30 g/L RS; C represents inoculation after AF; 2056 Lalvin L2056 yeast used for AF; α represents MLF starter culture Enoferm alpha; 41 represents Lalvin VP41 MLF starter culture. No histamine and tyramine was detected for the treatments A, as well as no histamine in control 2056. Each bar indicates the average of the replicates.

## 6.4 CONCLUSION

Results obtained confirmed previous research that if a compatible yeast and MLF starter culture are used there will be no influence on the alcoholic fermentation when co-inoculated. The co-inoculation also reduced the risks associated with the natural LAB present at the beginning of fermentation. This could be seen in the co-inoculated treatments where no histamine or tyramine was produced when compared to the standard practice (at the end or after alcoholic fermentation) were the natural LAB had a longer time to be part of the fermentation and this resulted in increased histamine and tyramine levels.

Future work to be done is the evaluation of the wines after MLF to determine possible differences in wine aroma and volatile compounds to determine the over-all quality of wine produced by the different inoculation strategies. Also to evaluate the risks of VA production, when early inoculation of bacteria is practiced under high pH conditions.

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# 7. GENERAL DISCUSSION AND CONCLUSIONS

## 7.1 CONCLUDING REMARKS

Wine is a complex medium consisting of a spectrum of flavours, aromas and other organoleptic properties, when combined have to result into a high quality product. The definition of a quality wine was always in the hands of the winemaker, however today with globalisation and the worldwide access to information resulted in more knowledgeable consumers with a greater understanding of product value and what wine quality entails. Consumer preference therefore will have to be the barometer for production decisions as well as for the marketing of wines (Bisson *et al*., 2002). Control over the production of wine is of the utmost importance if the latter is to be pursued and successfully executed.

One of the important steps in the winemaking process that needs to be managed in order to ensure high quality of the end-product is malolactic fermentation (MLF). MLF in wine is a secondary fermentation that usually occurs at the end of alcoholic fermentation (AF). MLF refers to the biological process of wine deacidification in which the dicarboxylic L-malic acid (malate) is converted to the monocarboxylic L-lactic acid (lactate) and carbon dioxide (Davis *et al*., 1985). Besides the decrease in acidity, it also improves the microbiological stability and the organoleptic characteristics of wines (Davis *et al*. 1988; Kunkee, 1991). These above mentioned organoleptic changes are due to secondary bacterial metabolisms (Lonvaud-Funel, 1999), such as the metabolism of carbohydrates and amino acids. The most important compounds apart from lactic acid that are formed are acetic acid, diacetyl (buttery flavour), acetoin, acetaldehyde, 2,3-butanediol, 2-acetolactate, 2-acetohydroxybutyrate, ethyl acetate and ethyl lactate (Fornachon and Lloyd, 1965; Henick-Kling *et al*., 1994; Kandler, 1983; Mascarenhas, 1984). Malolactic fermentation is conducted by lactic acid bacteria (LAB) that contains the malolactic enzyme (MLE). *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* are all genera of LAB that are present in wine. Towards the end of AF spontaneous MLF is mainly conducted by *Oenococcus oeni* (Van Vuuren and Dicks, 1993). Spontaneous MLF may occur any time during or several months after the completion of AF. Control of MLF entails the use of starter cultures to ensure the fast onset and execution of the process. These MLF starter cultures consist of pure culture *O. oeni* that were isolated from grapes or wine. Starter cultures used in South Africa are mostly isolated from grape growing regions of the northern hemisphere or cooler viticultural climates. The climate in South Africa is warm,

and therefore the resulting grapes differ in constitution resulting in different sugar and acidity levels. With this higher sugar level and lower acidity the pH's of South African wines are much higher that wines from cooler viticultural regions. A higher pH leads to increased populations of LAB species to grow in wine (Lonvaud-Funel, 1999), which can then influence the MLF starter cultures and the process of MLF.

Another important compound that is formed by LAB is biogenic amines (BA's). If large amounts of these amines are ingested, they can cause health problems, such as hypertension, respiratory distress, migraine and psychiatric disorders, such as schizophrenia (Buckland *et al*., 1997; Ten Brink *et al*., 1990). With consumers demanding healthier products and the legislation with regards to the regulation of these compounds in wine underway, knowledge of the levels of BA's are increasingly important.

This study therefore in general focused on the evaluation of MLF starter cultures in high pH red wines of South Africa and the determination of BA levels that can possibly occur.

During the first part of this study MLF starter cultures was stretched. We found that all the treatments were able to conduct and finish MLF, but that with stretching natural LAB has a greater chance in influencing the composition of the wine rendering it spoilt. The mother tank and lees treatments in most cases conducted the fastest MLF. The BA's occurred in all treatments, but varied in level according to cultivar and treatments. The identification of the MLF bacteria that was present at the end of MLF was not determined and could have aided the results greatly. From such information one could have deducted to what extent the natural LAB played a role in the treatments where the MLF bacteria was stretched. If the natural LAB does take part in the MLF it could happen that these bacteria perform MLF with success or off-flavours could develop. In the case of the former it could result into a more complex wine. In such a case the stretching of MLF bacteria by using mother tanks or lees inoculations could be useful when aiming to make a wine blend. But it remains a gamble and therefore the risks involving the stretching of MLF starter cultures cannot be stressed enough.

During the second part of this study MLF starter cultures were evaluated with and without the competition of natural occurring LAB and acetic acid bacteria (AAB) at different wine pH's. We found that in the case of the combination of the MLF starter culture with the *Lactobacillus* spp. their seemed to be a stimulation of MLF and caused a higher rate of malic acid degradation. This stimulation was speculated to exist due to competition with

regards to nutrient availability or it could be that their were more cells present than in the other treatments. Biogenic amines were present after MLF, but only putrescine and cadaverine were observed (no histamine and tyramine developed). The fact that inoculation of MLF starter cultures in combination with lower concentrations of other wine isolated bacteria (LAB and AAB) seem to enhance the performance of the starter cultures, opens the door to an array of studies to ensue. The LAB selected for this combination with starter cultures should be tested for their ability to perform MLF separately, the compounds that they develop and the overall influence of the organism on wine quality. Then the influence of the combination should also be tested to this regard. If successful combination of different LAB that influences wine positively could result into more complex MLF aromas in wines, enhancing wine quality. If possible commercial combinations could result from such work.

During the third part of this study the influence of the addition of commercial tannins and a pectolytic enzyme on MLF was evaluated. We found that in some instances there were slight inhibitory effects and in other cases stimulatory effects occurred. These effects were mostly present over the initial stages of MLF, but MLF was able to finish in all the treatments. The phenolic compounds that were measured changed during the process of MLF, mostly decreasing during this period. The BA levels that occurred showed an especially high level of putrescine in most of the treatments. A difference in BA levels was found between strains. Only one of the commercial tannin treatments led to production of histamine during this experiment. Further studies could entail the obtaining of cell numbers on a weekly basis. That could result in a more clear picture of the influence of the commercial tannin on the bacterial growth and not only the ability of the starter cultures to degrade malic acid. This study clearly showed that the addition of commercial tannins, even at elevated dosages, does not significantly inhibit or stimulate MLF. The use of commercial tannins is a common practice in the South African winemaking process and therefore all information with regards to the potential influence of these products on the wine is invaluable.

During the fourth part of this study inoculation time of MLF starter cultures was evaluated. We found that co-inoculation of MLF starter cultures and yeast before the start of AF resulted into a much faster MLF rate than inoculating starter cultures just before the end of AF or after AF. This was also the treatment that did not develop any histamine or tyramine. These results in my opinion can greatly aid winemakers on a practical level.

This fast MLF time could ensure the faster movement of wine through the cellar system to ease production flow and lessen logistical problems that often occur in the cellars. The only question that remains is if a faster MLF will lead to the loss of the aroma and character development in wine that occurs during MLF. If this is the case, co-inoculation will still find a use in the production of wines that is not meant as your premium range.

The results of this study provides a wide rage of knowledge with regards to the use of MLF starter cultures in high pH red wines of South Africa.

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