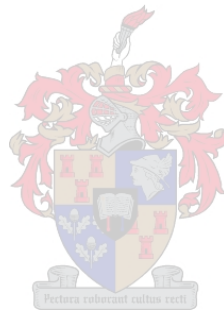


Assessing the compatibility and aroma production of NT 202 Co- Inoculant with different wine yeasts and additives

by

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Declaration

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Summary

The influence of malolactic fermentation (MLF) in most red and some white wines is one of many factors that determine or influence wine quality, because it affects the flavour and sensory profile of wine. This process is a decarboxylation process conducted by lactic acid bacteria (LAB) such as *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc*. Mostly *Oenococcus oeni*, but recently also *Lactobacillus plantarum* is used in commercial starter cultures and also the first mixed MLF starter culture (NT 202 Co-Inoculant) was commercialized in 2011. The reason for the predominant use of *O. oeni* and recently *L. plantarum* is due to their tolerance to the harsh wine environment.

Malolactic fermentation leads to a decrease in acidity and an increase in pH that leaves the wine with a softer mouthfeel. Another reason to conduct MLF is the improvement of microbial stability by the removal of malic acid as carbon source. Research focus has recently shifted to the ability of LAB and MLF as well as the interaction of LAB with yeast to alter the wine aroma profile via the modification and/or production of certain aroma compounds.

The main goal of this study was to assess the impact of yeast and nutrient addition on the ability of the NT 202 Co-Inoculant to conduct MLF during co-inoculation and to evaluate the aroma compound production in the final wine.

The first aim was to evaluate the impact of different red and white wine yeast strains on the ability of the NT 202 Co-Inoculant to conduct MLF during co-inoculation in Chardonnay, Merlot and Shiraz. Malolactic fermentation was unsuccessful in the Chardonnay due to a low pH, but successful in Merlot and Shiraz. Based on the malic acid degradation ability of the NT 202 Co-Inoculant, the yeasts were grouped into three categories: inhibitory, neutral or stimulatory towards MLF. Co-inoculated MLF showed a clear decrease in total fermentation time while yeast strains such as WE 372 and Exotics showed positive compatibility with the NT 202 Co-Inoculant. The impact of the yeast-bacterial combinations on the aroma compound production in the final wine was evaluated. Co-inoculated MLF showed positive aroma changes in the red wines with a general increase in total esters (associated with fruity characters in wine) especially ethyl lactate and diethyl succinate that also contribute to the mouthfeel of the wine. Production of esters, volatile fatty acids and higher alcohols seemed to depend on the yeast- and LAB strain used. The NT 202 Co-Inoculant contributed to the monoterpenes produced and MLF led to increased concentrations of diacetyl and acetoin, which are associated with buttery characters in wine.

The second aim of this study was to evaluate the impact of wine additives (used during co-inoculation) such as yeast- and bacterial nutrients, clarifying- and detoxifying agents on the ability of the NT 202 Co-Inoculant to conduct MLF and to assess their impact on the aroma compound production in the final wine. No negative or positive impact on the malic acid degradation of the NT 202 Co-Inoculant or the resulting aroma compound production was observed for the different wine additives used in this study.

The results generated from this study showed that the selection of yeast strains is important as it will influence both the fermentation duration and final wine aroma.

Opsomming

Die invloed van appelmelksuurgisting (AMG) in die meeste rooi- en witwyne is een van baie faktore wat wynkwaliteit beïnvloed, omrede dit die geur en sensoriese profiel van wyn beïnvloed. Hierdie proses is 'n dekarboksileringsaksie wat deur melksuurbakterieë (MSB), soos *Oenococcus*, *Lactobacillus*, *Pediococcus* en *Leuconostoc*, uitgevoer word. Die mees algemene bakterieë wat gebruik word, is *Oenococcus oeni*, maar onlangs het *Lactobacillus plantarum* ook na vore getree in die gebruik van kommersiële aanvangskulture. Die eerste gemengde AMG-aanvangskultuur (NT 202 Co-Inoculant) is in 2011 gekommersialiseer. Die rede vir die oorheersende gebruik van *O. oeni* en *L. plantarum* word toegeskryf aan hul gehardheidsgraad in 'n uitdagende wynomgewing.

Appelmelksuurgisting lei tot 'n afname in die suurheidsgraad en 'n toename in die pH van die wyn, wat 'n sagter mondgevoel tot gevolg het. Nog 'n rede waarom AMG deurgevoer word, is om die mikrobiële stabiliteit van die wyn te verbeter deur die verwydering van appelsuur as koolstofbron. Die navorsingsfokus het onlangs verskuif na die vermoë van MSB en AMG, sowel as die interaksie van MSB met die gis, om die wynaromaprofiel te verander deur middel van die verandering en/of produksie van sekere aromaverbindings.

Die hoofdoel van hierdie studie was om die impak van die gis en voedingstof te evalueer ten opsigte van die vermoë van die NT 202 Co-Inoculant om AMG uit te voer tydens koïnokulasie. Die produksie van aromakomponente in die finale wyn is ook geëvalueer.

Die eerste doelwit was om die impak van verskillende rooi- en witwyngisrasse te evalueer ten opsigte van die vermoë van die NT 202 Co-Inoculant om AMG uit te voer tydens koïnokulasie in Chardonnay, Merlot en Shiraz. Appelmelksuurgisting was onsuksesvol in die Chardonnay weens 'n lae pH, maar suksesvol in Merlot en Shiraz. In terme van die appelsuurafbraakvermoë van die NT 202 Co-Inoculant, is die giste in drie kategorieë gegroepeer: inhiberend, neutraal of stimulerend teenoor AMG. Ge-koïnokuleerde AMG het 'n duidelike afname in die totale fermentasietyd getoon, terwyl gisrasse, soos WE 372 en Exotics, 'n positiewe verenigbaarheid met die NT 202 Co-Inoculant getoon het. Die impak van die gis-bakteriële kombinasies op die aromakomponentproduksie in die finale wyn is geëvalueer. Ge-koïnokuleerde AMG het positiewe aromaveranderinge in die rooiwyne getoon met 'n algemene toename in die totale esters (wat geassosieer word met vrugtige karakters in wyn), veral etiellaktaat en dietielsuksinaat, wat ook bydra tot die mondgevoel van die wyn. Dit het voorgekom dat produksie van esters, vlugtige vetsure en hoër alkohole moontlik afhanklik kan wees van die gis- en bakteriële ras gebruik. Die NT 202 Co-Inoculant het bygedra tot die monoterpene wat geproduseer is en AMG het gelei tot verhoogde konsentrasies van diasetiel en asetoëne, wat geassosieer word met botteragtige karakters in wyn.

Die tweede doelwit van hierdie studie was om die impak van wyntoevoegingsmiddels (wat tydens koïnokulasie gebruik word) bv. gis- en bakteriële voedingstowwe, verhelderingsagente,

asook detoksifiserende agente, te evalueer ten opsigte van die vermoë van die NT 202 Co-Inoculant om AMG uit te voer en om hul impak op die produksie van die aromakomponente van die finale wyn te ontleed. Geen negatiewe of positiewe effekte is waargeneem vir die verskillende wyntoevoegingsmiddels, wat in hierdie studie gebruik is, in terme van die appelsuurafbraak van die NT 202 Co-Inoculant of die gevolglike produksie van aromakomponente nie.

Hierdie studie se resultate toon dat die keuse van die gisras belangrik is, omdat dit die fermentasietydperk, asook die finale wynaroma, beïnvloed.

This thesis is dedicated to my loving parents and sister who have always encouraged and supported me.

Hierdie tesis word opgedra aan my liefdevolle ouers en suster wat my nog altyd aangemoedig en ondersteun het.

Biographical sketch

Marené Schöltz was born on May 25th, 1987 in Newcastle, Kwa-Zulu Natal and matriculated at Bellville High School in 2005. Marené obtained her BScAgric degree (Viticulture & Oenology) at Stellenbosch University in 2010. In 2011 she enrolled at the same University for a Masters degree in Oenology.

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Preface

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately and is written according to the style of the South African Journal of Enology and Viticulture.

Chapter 1 **Introduction and project aims**

Chapter 2 **Literature review**
Malolactic fermentation: A mini review

Chapter 3 **Research results**
Impact of yeast and nutrient addition on the NT 202 Co-Inoculant mixed MLF starter culture and the aroma compound production in the final wine

Chapter 4 **General discussion and conclusions**

Table of Contents

Chapter 1. General introduction and project aims	1
1.1 Introduction	2
1.2 Project aims	4
1.3 Literature cited	5
Chapter 2. Literature review	8
Malolactic fermentation: A mini review	
2.1 Introduction	9
2.2 Factors that influence LAB growth and MLF	10
2.2.1 Yeast-bacteria interactions	12
2.2.2 Nutrient additions	13
2.3 Inoculation regimes	15
2.3.1 Sequential inoculation	16
2.3.2 Mid-AF inoculation	16
2.3.2 Co-inoculation	16
2.4 Commercial cultures	17
2.4.1 <i>Oenococcus oeni</i>	18
2.4.2 <i>Lactobacillus plantarum</i>	19
2.5 Aroma modification	20
2.5.1 Esters	21
2.5.2 Higher alcohols	22
2.5.3 Volatile fatty acids	22
2.5.4 Carbonyl compounds	23
2.5.5 Monoterpenes	25
2.5.6 Volatile sulphur compounds	25
2.6 Concluding remarks	26
2.7 Literature cited	27
Chapter 3. Research results	34
Impact of yeast and nutrient addition on the NT 202 Co-Inoculant mixed MLF starter culture and the aroma compound production in the final wine	
3.1 Introduction	35
3.2 Materials and methods	37

3.2.1	Vinification procedures	37
3.2.2	Treatments	37
3.2.3	Sampling	39
3.2.4	Microbial enumeration	39
3.2.5	Standard analyses	40
3.2.6	Volatile aroma compounds	41
3.2.7	Data analyses	42
3.3	Results and discussion	43
3.3.1	Fermentation kinetics	43
3.3.2	Microbial analysis	53
3.3.3	Volatile aroma compounds	59
3.4	Conclusions	98
3.5	Literature cited	99
Chapter 4. General discussion and conclusions		103
<hr/>		
4.1	Concluding remarks and future work	104
4.2	Literature cited	106

Chapter 1

Introduction and project aims

1. Introduction and project aims

1.1 Introduction

Wine is a complex medium that is the result of interactions between the grape matrix and microorganisms like fungi, yeasts and bacteria (Gao *et al.*, 2002; Fleet, 2003). High levels of ethanol, low pH and temperature as well as the presence of sulphur dioxide (SO₂) make wine a harsh environment for microorganisms to survive in (Pretorius, 2000; Comitini *et al.*, 2005; Du Toit *et al.*, 2011). Two main processes occur during vinification namely alcoholic fermentation (AF) and malolactic fermentation (MLF). Fermentations can be carried out spontaneously by the microorganisms naturally present or by inoculation with commercial starter cultures. Spontaneous fermentations rely on the natural microflora present on the grapes and the associated winery equipment to partake in the biochemical conversions associated with AF and MLF. If spontaneous MLF is desired, then MLF will usually follow the completion of AF and MLF will be carried out by the indigenous LAB present in the wine (Nielsen *et al.*, 1996).

Alcoholic fermentation, the conversion of sugar into ethanol with carbon dioxide (CO₂) as by-product, is carried out by yeast, mostly *Saccharomyces cerevisiae* (Pretorius, 2000; Fleet, 2003; Alexandre *et al.*, 2004). The lack of control over which yeast strain dominates AF is what adds to the risk of doing spontaneous AF. Yeasts require a range of nutrients to optimally grow during fermentation. Yeast nutrient requirements include carbon (sugars), nitrogen (ammonia and/or amino acids), and various growth and survival factors such as minerals and vitamins (Fugelsang and Edwards, 2007).

Malolactic fermentation occurs in most red- and some white wines as a secondary fermentation (Lonvaud-Funel, 1999; Alexandre *et al.*, 2004; Lerm *et al.*, 2010; Du Toit *et al.*, 2011; Abrahamse and Bartowsky, 2012). Lactic acid bacteria (LAB), such as *Oenococcus oeni* and *Lactobacillus* species drive MLF, the decarboxylation of L-malic acid to L-lactic acid and CO₂ (Nehme *et al.*, 2010; Du Toit *et al.*, 2011; Abrahamse and Bartowsky, 2012). Four genera of LAB have been identified as being involved in winemaking: *Pediococcus*, *Leuconostoc*, *Oenococcus* and *Lactobacillus*, of which *O. oeni* is best adapted to survive the harsh wine environment (Liu, 2002; Du Toit *et al.*, 2011). This explains why *O. oeni* is more likely to dominate during MLF and is used in most MLF starter cultures (Du Toit *et al.*, 2011). *Lactobacillus* species have also proved that they can survive harsh wine conditions, especially in high pH wines (Du Toit *et al.*, 2011) and are therefore being implemented for use in MLF starter cultures.

Like yeast, LAB have complex nutrient requirements that generally include carbon, phosphate, manganese, amino acids (proline, arginine, valine, leucine and isoleucine), as well as vitamins (nicotinic acid and pantothenic acid) (Terrade and De Orduña, 2009). Terrade and De Orduña (2009) found that the two *O. oeni* strains tested were more fastidious than the *Lactobacillus* spp. tested. They found that riboflavin was the only vitamin required by only the

Lactobacillus spp. tested, whereas L-glycine, L-threonine, L-methionine, L-histidine, L-tyrosine and L-tryptophan were the only amino acids required by only the two *O. oeni* strains tested. These nutrient requirements become even more important if MLF is conducted after the completion of AF when the yeast has already utilized the nutrients present in the wine. Risks involved in spontaneous MLF include wine spoilage due to the production of off-flavours (acetic acid, mousiness and volatile phenols) and health implications due to the production of biogenic amines and ethyl carbamate (Chatonnet *et al.*, 1995; Costello *et al.*, 2001; Uthurry *et al.*, 2006; Landete *et al.*, 2007; Bartowsky and Henschke, 2008). The main reasons for conducting MLF are to de-acidify the wine, improve the wine aroma and improve microbial stability by removal of malic acid as a carbon source (Bartowsky and Pretorius, 2008; Lerm *et al.*, 2010; Abrahamse and Bartowsky, 2012). Different inoculation regimes can be used to conduct MLF. Inoculation with LAB can be done with the yeast (co-inoculation), mid AF or post AF (Lerm *et al.*, 2010). Co-inoculation has proved to be advantageous by reducing the overall fermentation time, allowing wines to be stabilized at an earlier stage (Lerm *et al.*, 2010; Abrahamse and Bartowsky, 2012) and providing efficient fermentation tank utilization in the cellar (Jussier *et al.*, 2006). A recent study done by Massera *et al.* (2009) on Malbec grape juice using co-inoculation, demonstrated positive outcomes with no negative impact on the yeast population or AF performance and no increase in biogenic amine formation. A study done by Jussier *et al.* (2006) showed no negative impact of co-inoculation on the fermentation success and kinetics or final wine parameters.

Interactions between the microorganisms involved during vinification can affect the final wine product in various ways. Yeast species may interact with one another as well as with the LAB present in the matrix. The effect of yeast on LAB have been proven to be either inhibitory, via the production of ethanol, SO₂, medium chain fatty acids and proteinaceous compounds (Comitini *et al.*, 2005; Osborne and Edwards, 2007; Mendoza *et al.*, 2010), or stimulatory of nature by releasing nutrients such as vitamins, amino acids, lipids, glucans, cell wall polysaccharides and proteins (Alexandre *et al.*, 2004; Muñoz *et al.*, 2011).

The microbiological profile of a wine can have positive and negative influences on wine flavour (Fleet, 2003). The advantageous effects of MLF on wine aroma have been well studied and usually include final wine descriptors such as buttery and nutty, whereas co-inoculation leads to less buttery and more fruity wines (Lerm *et al.*, 2010). Diacetyl is probably the most important compound regarding the buttery aroma and flavour characteristic associated with MLF. Citric acid metabolism by LAB leads to diacetyl and is formed as an intermediate in the reductive decarboxylation of pyruvic acid to 2, 3-pentadiol (Swiegers *et al.*, 2005).

The other important group of compounds associated with MLF are esters that drive fruitiness in wine and are normally increased by MLF (Lerm, 2010; Knoll *et al.*, 2011; Abrahamse and Bartowsky, 2012; Knoll *et al.*, 2012; Malherbe *et al.*, 2012). Two of the most important esters that play a role during MLF are ethyl lactate, an esterification product of ethanol present (due to AF) and lactic acid produced by LAB during MLF (Lerm *et al.*, 2010) and

diethyl succinate, formed via the non-enzymatic esterification of succinic acid (Ugliano and Moio, 2005). The beneficial characteristics that ethyl lactate provide to the wine aroma profile include descriptors such as fruity, creamy and buttery as well as a contribution to the mouthfeel of the wine (Lerm *et al.*, 2010), whereas diethyl succinate imparts fruity aromas to the wine (Peinado *et al.*, 2004).

Among the *Lactobacillus* species, *Lactobacillus plantarum* proved to have favourable β -glucosidase activity (Michlmayr *et al.*, 2010; Mtshali *et al.*, 2010) that can modify the sensorial profile of the wine by hydrolysing sugar-bound monoterpenes to release volatile, aromatic monoterpenes (Liu, 2002; Mtshali *et al.*, 2010).

A range of additives can be added to wine for various reasons. Such additives include yeast- and bacterial nutrients, detoxifying- and clarification agents. Nutritional additives that contain inactivated yeasts can provide organic nitrogen, trace elements, available amino nitrogen, phosphates, cell wall polysaccharides, cellulose (to provide a surface to adsorb toxic compounds and keep LAB in suspension), mineral cofactors as well as vitamins. Mannoprotein addition for the purpose of clarification in wine has an influence on LAB. Mannoproteins released during autolysis or AF can adsorb medium chain fatty acids that inhibit LAB growth (Alexandre *et al.*, 2004). A study done by Diez *et al.* (2010) found that yeast commercial mannoproteins of intermediate molecular weight (6-22kD) increased *O. oeni* growth in the presence of ethanol. Yeast hulls (ghost yeasts) that are added to detoxify wines can also serve as bacterial nutrients and aid in successful MLF by reducing antagonism by growing yeast (Du Toit *et al.*, 2011).

There is a lack of information on the impact of yeast nutrient addition and the addition of clarifying- and detoxifying agents on LAB and MLF in wine, especially when using a MLF starter culture that comprises of *O. oeni* and *L. plantarum*.

1.2 Project aims

The primary aim of this study was to assess the compatibility and aroma compound production of the mixed MLF starter culture consisting of *O. oeni* and *L. plantarum* called NT 202 Co-Inoculant (Anchor Yeast) in co-inoculation with commercial wine yeast strains. The second aim of this study was to evaluate the impact of yeast nutrient-, bacterial nutrient-, detoxifying agent- and clarification agent addition on the final wine aroma when used in combination with the NT 202 Co-Inoculant.

The specific aims of the study were as follow:

- (i) to assess the impact of different white wine yeast strains on the ability of the NT 202 Co-Inoculant (Anchor Yeast) to conduct MLF compared to Viniflora CH35 (Christian Hansen) and Lalvin VP41 (Lallemand) in Chardonnay in co-inoculation during the 2011 vintage;

- (ii) to assess the MLF compatibility of 14 red wine yeast strains in co-inoculation with commercial MLF starter cultures, NT 202 Co-Inoculant, Viniflora oenos (Christian Hansen) and Lalvin VP41, in Merlot 2011 and Shiraz 2012;
- (iii) to determine the major volatile aroma compounds, monoterpenes and principal carbonyl compounds after completion of MLF in the 2011 Merlot as well as the 2012 Shiraz using gas chromatographic techniques;
- (iv) to assess the impact of commercial additives, such as nutrients, detoxifying- and clarification agents when used in co-inoculation on MLF kinetics as well as on the aroma compounds produced using gas chromatographic techniques; and
- (v) to do multivariate data analysis on all generated data sets.

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Chapter 2

Literature review

Malolactic fermentation: A mini review

2. Literature review

Malolactic fermentation: A mini review

2.1 Introduction

Alcoholic fermentation (AF) is the primary fermentation in wine, carried out by yeast, mainly the more alcohol tolerant *Saccharomyces cerevisiae* that convert sugar to ethanol and CO₂ (Pretorius, 2000; Matthews *et al.*, 2004). Other yeast genera frequently associated with wine include *Torulaspora*, *Candida*, *Hanseniaspora*, *Brettanomyces*, *Pichia*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Willopsis* and *Kloeckera*, to name a few (Pretorius, 2000; Jolly *et al.*, 2006; Zott *et al.*, 2010; Comitini *et al.*, 2011). Alcoholic fermentation, especially choice of yeast strain, contributes to the aroma profile of the wine by producing compounds such as esters, higher alcohols, aldehydes and fatty acids (Swiegers *et al.*, 2005; Dubourdieu *et al.*, 2006; Styger *et al.*, 2011). According to Swiegers and Pretorius (2007), wine yeasts are the main producers of volatile sulphur compounds, generated from sulphur sources [some cases even sulphur dioxide (SO₂) added by winemakers] and grape-derived precursors. The increase in some monoterpenes (such as geraniol and linalool) after fermentation might be due to β -glucosidase activity of the yeast and/or chemical hydrolysis of the bound forms (Lambrechts and Pretorius, 2000; Mateo and Jiménez, 2000; Carrau *et al.*, 2005). On the other hand, a study done by Carrau *et al.* (2005) showed that monoterpene biosynthesis by yeast, associated with floral aroma in wine, can be of *de novo* origin.

Malolactic fermentation (MLF) is a secondary fermentation conducted by lactic acid bacteria (LAB), mainly *Oenococcus oeni*, in most red- and some white- and sparkling wines (Lerm *et al.*, 2010). It is a decarboxylation process where L-malic acid is converted to L-lactic acid with the production of CO₂ (Solieri *et al.*, 2010). The three main reasons for conducting MLF in wine are: to deacidify the wine, to improve microbial stability of the wine by removing malic acid (malate) as a possible carbon source and to modify wine aroma (Maicas *et al.*, 1999; Liu, 2002; Bartowsky and Borneman, 2011; Knoll *et al.*, 2011). Malolactic fermentation can modify wine aroma via the production or modification of flavour-active compounds (Swiegers *et al.*, 2005; Boido *et al.*, 2009; Michlmayr *et al.*, 2012). In cooler climate countries such as New Zealand and Canada that produce high acid wines, MLF is mostly conducted for the purpose of deacidification (Liu, 2002). In warmer regions, where deacidification is of less importance as lower malic acid concentrations are present in the grapes, MLF is mainly conducted for the purpose of changing the sensorial profile of the wine (Lerm, 2010).

The LAB responsible for MLF in wine is mostly of the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Lonvaud-Funel, 1999; Muñoz *et al.*, 2011). Of the four genera LAB found in wine, *O. oeni* is possibly the best adapted to overcome high ethanol levels, low pH and temperatures as well as SO₂ that make wine a harsh environment (Du Toit *et al.*, 2011).

This explains the use of *O. oeni* as the predominant LAB in MLF starter cultures today. *Lactobacillus plantarum*, however, have also proven its resilience and have therefore been included in a commercial MLF starter culture, together with *O. oeni* (Lerm *et al.*, 2011).

The focus of this mini literature review will be to summarise the most important aspects associated with MLF. Various factors influence MLF of which yeast-bacteria interactions and the addition of nutrients will be discussed in more detail. Different inoculation times, the use of commercial starter cultures, as well as the impact of MLF on wine aroma will also be discussed.

2.2 Factors that influence LAB growth and MLF

In the complex, harsh wine environment containing different microorganisms that compete for survival, many factors can influence LAB growth and therefore successful completion of MLF. These factors include high ethanol concentration (can exceed 15% v/v), low pH (can be less than 3.2), low temperature and SO₂ concentration (can be more than 50 mg/L), lysozyme, phenolic compounds, medium chain fatty acids, yeast-bacteria interactions and nutrient availability (Guerzoni *et al.*, 1995; Carreté *et al.*, 2002; Gao *et al.*, 2002; Rosi *et al.*, 2003; Alexandre *et al.*, 2004; Campos *et al.*, 2009; Diez *et al.*, 2010; Bartowsky and Borneman, 2011; Knoll *et al.*, 2012; Quirós *et al.*, 2012). A study by Costello *et al.* (2012) found that the extent and diversity of the impact of MLF on wine sensory and chemical properties are influenced by the choice of LAB strain, pre-MLF pH and wine matrix composition.

Ethanol plays a critical role in the success of MLF, because it can disrupt membrane structures and affect many membrane associated processes, including malolactic activity and those involved in stress resistance (Da Silveira *et al.*, 2003; Chu-Ky *et al.*, 2005; Zapparoli *et al.*, 2009). According to Rosi *et al.* (2003), ethanol and pH are the most important wine parameters impacting on bacterial activity. In their study they found that pH values below 3.2 lowered *O. oeni* viability. A study done by Zapparoli *et al.* (2009) confirmed that high ethanol and low pH are two stress factors that influence the survival of LAB, and thus MLF, when combined with other oenological factors. Ethanol has also shown synergistic interactions with temperature to inhibit LAB growth (Lerm, 2010). High ethanol concentrations lower the optimal growth temperature of LAB whereas increased temperatures lower the tolerance of LAB to endure higher ethanol concentrations (Henick-Kling, 1993). In a review by Wibowo *et al.* (1985), it is stated that the ability of LAB to survive and grow in wine decreases as the alcohol concentration increases above 10% (v/v). In the presence of 10% to 14% (v/v) ethanol, the optimal growth of LAB is between 18 and 20°C, whereas optimum growth at 30°C is achieved at only 0% to 4% (v/v) (Henick-Kling, 1993).

The effect of SO₂ on LAB is dependent on factors including yeast strain and wine composition, specifically wine pH (Alexandre *et al.*, 2004). It has been found that it is the molecular form of SO₂ that is toxic to wine yeasts and bacteria (Nehme *et al.*, 2008). Nehme *et al.* (2008) also suggested that the molecular SO₂ inhibits bacterial growth by reducing

maximal biomass and the malic acid activity. As wine pH decreases, the amount of molecular SO₂ increases and *vice versa* (Nehme *et al.*, 2008).

In literature it has been found that lysozyme and phenolic compounds can inhibit LAB (Gao *et al.*, 2002; Campos *et al.*, 2009). Cabrita *et al.* (2008) stated that some phenolic acids inhibit LAB growth while others stimulate MLF carried out by *O. oeni*. Diez *et al.* (2010) found that malvidin, an anthocyanin, activated the growth of some LAB strains only in the presence of 6% ethanol. Reguant *et al.* (2000) found that MLF was progressively delayed with increasing levels of *p*-coumaric acid, but stimulated in the presence of catechin and quercetin. In contrast to this, Rozès *et al.* (2003) found that *O. oeni* growth was slightly stimulated by the presence of malvidin-3,5-diglucoside or by the mixture of phenol carboxylic acids (caffeic, ferulic, *p*-coumaric and gallic acids) and catechin. Campos *et al.* (2009) found that, with the exception of gallic acid, all tested phenolic acids negatively affected the growth rate of *Lactobacillus hilgardii* and *O. oeni*, but more so in the case of *O. oeni*, indicating that the effect of phenolic acids on LAB is species or strain dependent. They also found that for *L. hilgardii*, all phenolic acids, except gallic acid, extended the completion time for MLF. Gao *et al.* (2002) examined the impact of lysozyme on the cells and the cell counts of the four LAB cultures tested. Using a scanning electron microscope they observed that lysozyme had a detrimental effect on the cells of the LAB cultures, while cell counts (cfu/mL) indicated a dramatic decrease as soon as 125 or 250 mg/L of lysozyme was added, causing an 8 log reduction in some treatments. Such reductions in cell counts may cause a sluggish or even stuck MLF. A study by Guzzo *et al.* (2011) focused on the inhibitory effects of wine phenolics on lysozyme activity against LAB and found that phenolics reduced the inhibitory action of lysozyme against LAB, especially for *O. oeni*, which is more sensitive to lysozyme than *L. plantarum*.

Yeast can produce medium chain fatty acids, such as decanoic acid that impact on both growth rate and malolactic activity of LAB, depending on concentration, but also on the pH of the medium (Carreté *et al.*, 2002; Alexandre *et al.*, 2004). Mendoza *et al.* (2010) determined that high levels of dodecanoic acid (20 mg/L) inhibited *O. oeni* and *L. hilgardii*, but that bacterial growth was not affected by decanoic acid. They went on to state that the yeast strains they used in the study were poor producers of fatty acids thereby implying that production of medium chain fatty acids is also yeast strain dependent. Carreté *et al.* (2002) concluded from their study that fatty acids such as decanoic- and dodecanoic acids affected the ATPase activity of *O. oeni*, which might have caused their loss in viability. This loss in viability can affect MLF rate. Therefore, not only can medium chain fatty acids cause yeast-bacterial antagonism, but it can also reduce the malic acid degradation abilities of the bacteria (Alexandre *et al.*, 2004).

2.2.1 Yeast-bacteria interactions

Winemakers face the challenge of choosing compatible yeast and bacterial starter cultures for successful AF and MLF, but especially during co-inoculation. Winemaking practices and choice of starter cultures are two aspects that can influence yeast-bacteria interactions and therefore MLF (Nehme *et al.*, 2008). Winemakers can control these two aspects, thereby improving their fermentation management. The interaction between the yeast and bacteria will have a direct impact on the growth of LAB and therefore MLF. Many studies have been done on the interaction between yeast and bacteria to better understand this relationship (Fleet, 2003; Alexandre *et al.*, 2004; Comitini *et al.*, 2005; Jussier *et al.*, 2006; Nehme *et al.*, 2008; Aredes Fernández *et al.*, 2010; Mendoza *et al.*, 2010). Yeast-bacteria interaction can be inhibitory, neutral or stimulatory (Patynowski *et al.*, 2002; Comitini *et al.*, 2005) and depend on the choice of yeast and bacterial strain (Nehme *et al.*, 2008), the uptake and release of nutrients by the yeast and the ability of the yeast to produce metabolites that will affect the growth of LAB and therefore MLF (Alexandre *et al.*, 2004).

During AF, yeast produces metabolites that can be inhibitory or stimulatory towards LAB and MLF (Fleet, 2003; Alexandre *et al.*, 2004). Alexandre *et al.* (2004) reported on the main factors relating to the inhibition or stimulation of LAB by wine yeast. Most important factors were found to be dependent on the yeast strain used. A summary of what they reported will follow. The main factors relating to yeast that cause the inhibition of LAB include: competition for nutrients, production of ethanol, SO₂, medium chain fatty acids, and protein compounds. During yeast autolysis nutrients, favourable to bacterial growth, are released (Fleet, 2003). These metabolites can inhibit or stimulate LAB growth either as single compounds or synergistically.

Ethanol seems to rather reduce LAB growth than malolactic activity of the LAB (Nehme *et al.*, 2008). The ability of yeast to produce SO₂ depends on the strain used and the wine composition (Nehme *et al.*, 2008). Most commercial wine yeast strains are selected for their low SO₂ production and produce less than 30 mg/L SO₂. It has been reported that some strains produce more than 100 mg/L (Nehme *et al.*, 2008). Comitini *et al.* (2005) suggested possible synergistic effects, such as ethanol and SO₂, on the viability of LAB. A study done by Mendoza *et al.* (2010) revealed that ethanol, SO₂, or both metabolites together were not the only factors contributing to bacterial inhibition at the concentration tested.

Fatty acids have been shown to inhibit bacterial growth, but can be removed by adsorption to yeast cell walls to improve bacterial growth (Diez *et al.*, 2010). Carreté *et al.* (2002) suggested that the loss in viability may be due to inhibition of ATPase activity of *O. oeni* and that this ATPase activity is affected by ethanol, copper, fatty acids, especially dodecanoic acid, as well as SO₂.

A study by Comitini *et al.* (2005) found that a *S. cerevisiae* wine strain produced a proteinaceous factor that was able to inhibit *O. oeni* growth and MLF (Nehme *et al.*, 2010), but that nutrient depletion was not responsible for bacterial inhibition. In contrast to this, Nehme

et al. (2008) reported that the inhibition of LAB can result from nutrient depletion. A study by Osborne and Edwards (2007) found that the inhibition of *O. oeni* by a peptide (Mendoza *et al.*, 2010) seemed to depend on the presence of SO₂, but stated that because the antibacterial protein observed in the study done by Comitini *et al.* (2005) was not characterized, it is unknown whether these proteinaceous compounds were different. Osborne and Edwards (2007) also suggested that the antibacterial peptide enhanced the toxicity of SO₂ by disrupting the bacterial cell membrane thereby allowing SO₂ to enter the cell more easily. A study by Nehme *et al.* (2008) showed that the inhibition of MLF, in terms of malic acid consumption rate, exerted by *S. cerevisiae* was mainly due to ethanol and a peptidic fraction that has a MW between 5 and 10 kDa. Despite the inhibition observed in this study, co-inoculation of LAB and yeast was considered effective for MLF, but dependent on the choice of yeast and bacterial strains used. Nehme *et al.* (2010) concluded from their results that the inhibitory peptides are most likely strain dependent.

The stimulation of LAB by wine yeast are mostly due to yeast autolysis during yeast lees contact that provide autolysates containing nutrients that can stimulate LAB growth and therefore MLF. Such autolysates include nitrogenous compounds, such as amino acids, peptides and proteins (like mannoproteins), yeast macromolecules, such as cell wall polysaccharides, vitamins, nucleotides and lipids such as long chain fatty acids. The latter three factors have not been well studied. A study by Diez *et al.* (2010) found that mannoproteins stimulated *O. oeni* growth in the presence of ethanol and that the phenomenon was strain dependent.

Due to the possible antagonistic interaction between yeast and bacteria, it is important to choose compatible yeast and bacterial strains to conduct successful MLF.

2.2.2 Nutrient additions

Lactic acid bacteria are complex organisms and like yeast they require a range of nutrients for optimal growth and metabolism. These nutrients include vitamins, amino acids, which are essential for LAB metabolism and survival (Nehme *et al.*, 2008) as well as sugars, peptides, organic acids (malate, citrate and pyruvate), fatty acids, nucleic acids, minerals and trace elements (Mn, Mg, K and Na) (Krieger, 2006). In a study by Terrade and De Orduña (2009) it was found that all the tested strains of *Oenococcus* and *Lactobacillus* required 10 compounds and that their essential nutrient requirements were strain specific. The 10 compounds include a carbon and phosphate source, manganese, several amino acids (proline, arginine and the branched amino acids valine, leucine and isoleucine) and vitamins (nicotinic acid and pantothenic acid).

After completion of AF, wine may lack nutrients such as essential amino acids, thereby requiring nutrient additions to satisfy LAB nutrient requirements for optimal growth and functioning if MLF is desired (Remize *et al.*, 2006). The addition of nutrients to stimulate growth

reduces the competition for nutrients between yeast and bacteria, because yeast competes for sugars, amino nitrogen, vitamins, essential minerals and fatty acids (Henick-Kling *et al.*, 2004). It is important to keep in mind that yeast nutrient additions may also serve as nutrient supplements for the LAB and may therefore impact their growth and MLF. Therefore, careful consideration should be given to nutrient additions during co-inoculation when yeast and bacteria will compete for nutrients simultaneously.

Commercial yeast nutrient additives include diammonium phosphate (DAP), nutrient blends (some of which may contain DAP, also vitamins, nucleic acids and trace elements) and yeast extract (Henick-Kling *et al.*, 2004).

Most of the commercial bacterial nutrient additives consist of yeast extracts or yeast hulls/ghosts that contain amino acids, fatty acids, nucleic acids, vitamins and minerals (Henick-Kling *et al.*, 2004). Due to the risk of biogenic amine formation from certain amino acids (such as arginine that leads to putrescine formation), commercial nutrient additives usually include low amounts of these specific amino acids (Fugelsang and Edwards, 2007).

Some nutrients serve other purposes too, e.g. yeast hulls/ghosts that are also used as detoxifying agents. Besides yeast and bacterial nutrients, mannoproteins can be added to wine as a clarifying agent that may also impact LAB growth and/or MLF, because it eliminates a part of the native microflora (Guzzo and Desroche, 2009).

Diammonium phosphate (DAP) is not a nitrogen source and LAB cannot utilize ammonia and must therefore rely on amino acids (Fugelsang and Edwards, 2007). This means that DAP addition alone is not always sufficient to ensure successful MLF, thereby explaining the reason for additional nutrients, besides DAP, in some commercial nutrient additives.

As mentioned before, yeast extract and yeast hulls/ghosts provide the same type of nutrients, but yeast hulls/ghosts can also serve as a detoxifying agent, because it can help bind fungicides and antimicrobial peptides to the cell membrane and cell wall fragments (Henick-Kling *et al.*, 2004). Yeast hulls/ghosts can also absorb wine contaminants, such as anisoles, and have a high polysaccharide capacity. In a study by Munoz and Ingledew (1989) they found that yeast hulls/ghosts can adsorb to the toxic medium chain fatty acid decanoic acid.

Inactivated yeast are rich in amino acids, organic nitrogen, trace elements and vitamins (that serve as cofactors during MLF) and provide cell wall polysaccharides and cellulose. Polysaccharides can form complexes with tannins, which can inhibit LAB by inhibiting enzyme activity, adhere to cell walls or form complexes with copper and iron (Vivas *et al.*, 2000). Cellulose serves as an inert surface to which LAB can adhere during MLF to stay in suspension as well as a fining agent for bacterial inhibitors.

Mannoproteins (a family of polysaccharides) (Diez *et al.*, 2010) originate from yeast cell walls and are released from the yeast cells in the beginning of fermentation and during wine ageing on lees (Gonzalez-Ramos *et al.*, 2008). Mannoprotein additions (extracted from *S. cerevisiae*) are often used as clarifying agents, but may also impact bacterial growth.

Gonzalez-Ramos *et al.* (2008) stated that yeast mannoprotein additions can be used as oenological tools to stabilize the colour and sensorial properties of the wine. In a study by Diez *et al.* (2010) they found that yeast commercial mannoproteins of intermediate weight (6 – 22 kD) enhanced *O. oeni* growth (in 81.5% of the studied *O. oeni* strains) in the presence of ethanol. This study also found that mannoproteins can prevent acetic acid bacteria (AAB) growth, thereby contributing to microbiological control during winemaking.

To summarize, the synergistic inhibitory effects of ethanol, SO₂, fatty acids and reduced nutrient availability may only partly explain, but not clarify entirely, the inhibition in growth and malic acid degradation abilities of LAB (Nehme *et al.*, 2008). Certain protein compounds can also inhibit *O. oeni* (Comitini *et al.*, 2005; Osborne and Edwards, 2007). It is important for the winemaker to keep in mind these synergistic effects (such as relationship between pH and SO₂), as well as the effects of different additives to wine during the decision making processes to improve MLF and ultimately wine quality.

2.3 Inoculation regimes

Natural (or spontaneous or un-inoculated) MLF is generally considered to be carried out by the indigenous LAB present in the wine and/or on the winemaking equipment, making it very unpredictable (López *et al.*, 2011). It can be argued that the term 'un-inoculated' MLF is regarded as spontaneous MLF conducted in a cellar/winemaking space where MLF starter cultures have been previously introduced, thereby contributing to the LAB pool present in the cellar air or on the equipment.

Risks involved with spontaneous MLF include the possible presence of unidentified/spoilage microorganisms (such as AAB, spoilage strains of LAB and *Brettanomyces*) that can produce undesirable off-flavours and/or biogenic amines that can affect human health (Alexandre *et al.*, 2004; Lerm, 2010; López *et al.*, 2011), postponed onset or completion of MLF and bacteriophage infection of LAB (Lerm, 2010). All of these risks mentioned can diminish the wine quality.

Inoculation for MLF traditionally occurs after completion of AF (sequential inoculation) using commercial starter cultures (Massera *et al.*, 2009; Nehme *et al.*, 2010). Sequential inoculation is, however, not the only possible regime to conduct successful MLF. Inoculation of LAB can also be done halfway through AF (mid-AF) as well as with the yeast at the beginning of AF (co-inoculation/simultaneous) (Knoll *et al.*, 2012).

2.3.1 Sequential inoculation

Some literature suggests that sequential inoculation is a means to avoid problems associated with early inoculation such as antagonistic yeast-bacteria interactions (Lerm, 2010). Due to completion of AF, the lower residual sugar concentrations that reduces the risk of acetic acid production serves as another advantage of sequential inoculation (Costello, 2006).

Risks involved with sequential inoculation include sluggish or stuck MLF due to LAB viability problems caused by high ethanol concentrations, low pH, SO₂, other microbial compounds produced by the yeast and nutrient depletion (Larsen *et al.*, 2003). Massera *et al.* (2009) stated that inoculation with starter cultures after AF does not always result in dominance of the selected strain and the desired contribution.

2.3.2 Mid-AF inoculation

Some winemakers implement this inoculation regime to overcome high ethanol concentrations, as is the case with sequential inoculation, so the inoculated LAB can still adapt to the increasing ethanol concentrations. Other reasons why mid-AF inoculation may be implemented is, because most of the free SO₂ is bound, thereby reducing the possible inhibition of LAB by SO₂ and the heat generated from the on-going AF will aid in the MLF. A study by Rosi *et al.* (2003) showed an immediate and extreme decrease in LAB cell counts, when inoculated midway through AF, declining as low as 10⁴ cfu/mL in the first six to eight days after inoculation and increasing again to 10⁶ cfu/mL, at which point malic acid degradation began.

2.3.3 Co-inoculation

Co-inoculation of LAB and yeast is a helpful time saving tool that can be used in order to overcome high ethanol concentrations and reduced nutrient availability often associated with conditions after completion of AF leading to incomplete MLF (Jussier *et al.*, 2006). The gradual adaptations of the bacteria to the increasing ethanol concentrations enhance their performance (Zapparoli *et al.*, 2009). Co-inoculation allows an early dominance of the selected strain and better control over the outcome of MLF (Massera *et al.*, 2009). A study done by Jussier *et al.* (2006) in cool climate Chardonnay could not confirm a negative impact of co-inoculation compared to sequential inoculation on fermentation success and kinetics or on the final wine parameters. The same study found no sensorial differences between sequential and co-inoculation strategies followed or bacterial strain used in Chardonnay. A study done by Nehme *et al.* (2008) found improved bacterial growth and malic acid consumption using co-inoculation.

Possible yeast-bacterial interaction (as previously discussed) that might occur during co-inoculation is an important factor during decision making regarding inoculation time. Homofermentative LAB (such as *L. plantarum*) produces lactic acid as the major end product; whereas heterofermentative LAB (such as *O. oeni*) produce lactic acid, CO₂, ethanol and/or

acetic acid (Zúñiga *et al.*, 1993). The risk of increased volatile acidity due to sugar metabolism by bacteria is negligible if AF is successfully carried out by yeasts (Azzolini *et al.*, 2011). This statement is in agreement with a study done by Nehme *et al.* (2010) and Knoll *et al.* (2012) that showed no risk of increased volatile acidity during co-inoculation. The fear of this possible increase in volatile acidity is the reason for the sparse use of co-inoculation in the industry currently (Nehme *et al.*, 2010). Studies show that co-inoculation reduces the overall fermentation time without affecting AF (Massera *et al.*, 2009; Abrahamse and Bartowsky, 2012; Knoll *et al.*, 2012). Shortened fermentation times provide the opportunity to stabilize the wines earlier thereby reducing the risk of microbial spoilage (Abrahamse and Bartowsky, 2012). In the study done by Massera *et al.* (2009), co-inoculated MLF completed in 10 to 26 days without an increase in biogenic amine production. A study done by Knoll *et al.* (2012) showed that co-inoculation tended to increase ethyl and acetate esters.

Co-inoculation is therefore a handy tool that can be used to overcome possible problematic wine conditions like high initial sugar content of the grapes (often associated with warm climate countries such as South Africa) leading to high alcohol levels and insufficient nutrient availability that may lead to sluggish or stuck MLF when inoculated after AF. Co-inoculation can also be used for better tank utilization in the cellar as well as improved microbial stability, because it reduces overall fermentation time without the risk of off-flavours (Jussier *et al.*, 2006; Nehme *et al.*, 2010).

2.4 Commercial cultures

It is common practice to induce MLF with bacterial strains selected for their beneficial properties regarding wine quality (Jussier *et al.*, 2006). Commercial MLF starter cultures have been marketed in many forms since their development. Before the 1980's, most were in liquid form, then frozen and freeze-dried cultures were developed, leading to the development of direct inoculation starter cultures in the 1990's (Krieger-Weber, 2009; Zapparoli *et al.*, 2009; Lerm, 2010; López *et al.*, 2011). The use of direct inoculation cultures simplifies shipping, storage and use, which increase their popularity (Lerm, 2010). Stretching, a risky technique some winemakers implement to cut down on expenses, can imply the use of starter cultures below the recommended dosage, re-use of commercial starter cultures (as in a mother tank inoculation) or wine lees of which MLF has been completed (Lerm, 2010). The decreased populations of the inoculated bacteria allow possible spoilage organism development and MLF may not complete successfully. Contamination of other fermentation vessels from a contaminated mother tank and lack of control over MLF are two other risks involved with the stretching technique (Van der Merwe, 2007).

The different forms of bacterial cultures have different characteristics (optimal temperatures, pH, alcohol and total SO₂ tolerances) and preparation protocols that need to be

followed carefully according to manufacturer's instructions to ensure that the starter cultures' full potential are utilized.

Several important criteria should be considered when selecting LAB for possible use in commercial starter cultures. These include tolerance to low pH, high ethanol and SO₂ concentrations, LAB should show good growth characteristics under vinification conditions, compatibility with the selected yeast strain, the inability to produce biogenic amines, the ability to survive the production process, the lack of off-odour or off-flavour production and the production of aroma compounds that could potentially contribute to a desirable aroma profile (Lerm *et al.*, 2011; López *et al.*, 2011).

Strain selection procedure starts with LAB isolation from a spontaneous fermentation that exhibit natural selective pressures of the typical harsh wine conditions (low pH and temperature as well as high ethanol and SO₂ concentrations), followed by several screening procedures and trial vinifications (Bou and Powell, 2006; Solieri *et al.*, 2010).

Two LAB strains are currently used in commercial MLF starter cultures. They are *O. oeni* (mainly) and *L. plantarum* that contribute positively to the sensorial properties of wine (Diez *et al.*, 2010; Lerm *et al.*, 2011). Since spontaneous MLF is rendered too risky by most winemakers, numerous studies have been done in order to find other resilient LAB strains, besides *O. oeni*, for commercial use. Zapparoli *et al.* (2009) concluded from their study that the inoculation regime as well as the preparation of the bacterial starter culture determined the ease of MLF. They stated that the acclimatization of the bacterial cells to the wine-water solution is a vital step that impacts the success of MLF.

The advantages of using commercial MLF starter cultures provide better control, fermentation reliability, style predictability and repeatability, but even with inoculation, successful MLF is not guaranteed, especially under harsh wine conditions (Guerzoni *et al.*, 1995).

The two main LAB species used in commercial starter cultures will be discussed.

2.4.1 *Oenococcus oeni*

Oenococcus oeni (formerly known as *Leuconostoc oenos*) (Dicks *et al.*, 1995) exhibit various secondary metabolic activities during MLF that can modify the sensory properties of wine (Bou and Powell, 2006; Bartowsky and Borneman, 2011). Of all LAB found in wine, *O. oeni* has the greatest capacity to grow in low pH (prefers pH less than 3.5) and in the presence of 10% (v/v) ethanol (Muñoz *et al.*, 2011; Du Toit *et al.*, 2011). *Oenococcus oeni* strains vary in their ability to metabolize malic acid efficiently and contribute to desirable sensory properties of the wine. These are two important factors to consider during strain selection for commercial starter cultures (Bartowsky and Borneman, 2011). A study by Michlmayr *et al.* (2012) showed that glycosidases from *O. oeni* could improve the typical Riesling aroma. Riesling aroma is associated with abundant levels of desirable monoterpenes (Swiegers and Pretorius, 2005). Glycosidase has been of particular interest, since it is associated with monoterpene production

that imparts pleasant aromas described as e.g. floral or rose-like (Swiegers and Pretorius, 2005).

Oenococcus oeni is considered to be the commercial LAB strain best adapted to the harsh wine conditions. Bartowsky and Borneman (2011) reported that there is a recent growing interest in characterising *O. oeni* strains that are unique to specific geographical wine regions in order to enhance regionality in the wines. A so-called 'citrate negative' pure *O. oeni* starter culture has recently been developed that, according to the manufacturer, does not degrade citric acid into acetic acid, diacetyl and 2,3-butanediol. Some winemakers may not want a characteristic buttery aroma (associated with diacetyl production) in their wines. See **section 2.5.4** for a short discussion about carbonyl compounds of interest during MLF.

2.4.2 *Lactobacillus plantarum*

Research has indicated that different *Lactobacillus* species partake in MLF and that some species exhibit promising characteristics for use during MLF (Mtshali *et al.*, 2010; Du Toit *et al.*, 2011). *Lactobacillus plantarum* is one of these that have recently been incorporated in a mixed starter culture with *O. oeni* for commercial use due to its tolerance to the harsh wine conditions (high ethanol and SO₂ concentrations, pH higher than 3.5 and temperatures of ± 20°C). This strain has the ability to conduct MLF just as efficiently as *O. oeni* and possesses many enzyme encoding genes important for desirable aroma production) (Mtshali *et al.*, 2010; Du Toit *et al.*, 2011; Lerm *et al.*, 2011). Such enzymes include glycosidase, protease, esterase, phenolic acid decarboxylase and citrate lyase (Mtshali *et al.*, 2010; Du Toit *et al.*, 2011).

Beta-glucosidase activity in *L. plantarum* has been shown in a few studies (Sestelo *et al.*, 2004; Grimaldi *et al.*, 2005a; Lerm *et al.*, 2011). Lerm *et al.* (2011) found that *L. plantarum* displayed a more diverse enzyme profile than *O. oeni*, particularly the aroma-modifying enzymes β -glucosidase and phenolic acid decarboxylase. This implied the potential use of *L. plantarum* for wine aroma profile modifications and commercial starter cultures. A study done by Guerzoni *et al.* (1995) confirmed that *L. plantarum* is more resistant than *O. oeni* to the combined action of various stresses such as pH, temperature, ethanol and malate concentration, at least at an ethanol concentration of less than 6% (v/v). They suggested that *L. plantarum* is therefore more competitive at the beginning of AF.

The diverse choice of starter cultures available today will aid the winemaker in managing MLF and wine aroma. It is, however, important for the winemaker to decide on the preferred style of wine before selecting the starter culture.

2.5 Aroma modification

During MLF, the aroma and flavour of wines are influenced by LAB via the production of volatile metabolites and the modification of grape- and yeast derived aroma compounds as depicted in **Figure 2.1** (Swiegers *et al.*, 2005; Boido *et al.*, 2009; Michlmayr *et al.*, 2012).

There is an increased recognition that LAB such as *O. oeni* possesses an array of secondary metabolic activities during MLF, which can modify the sensorial properties of wine. These secondary activities include the metabolism of organic acids, polysaccharides, carbohydrates and amino acids, and several enzymes such as glycosidases, esterases and proteases, which generate volatile compounds well above their odour detection threshold (Bartowsky and Borneman, 2011).

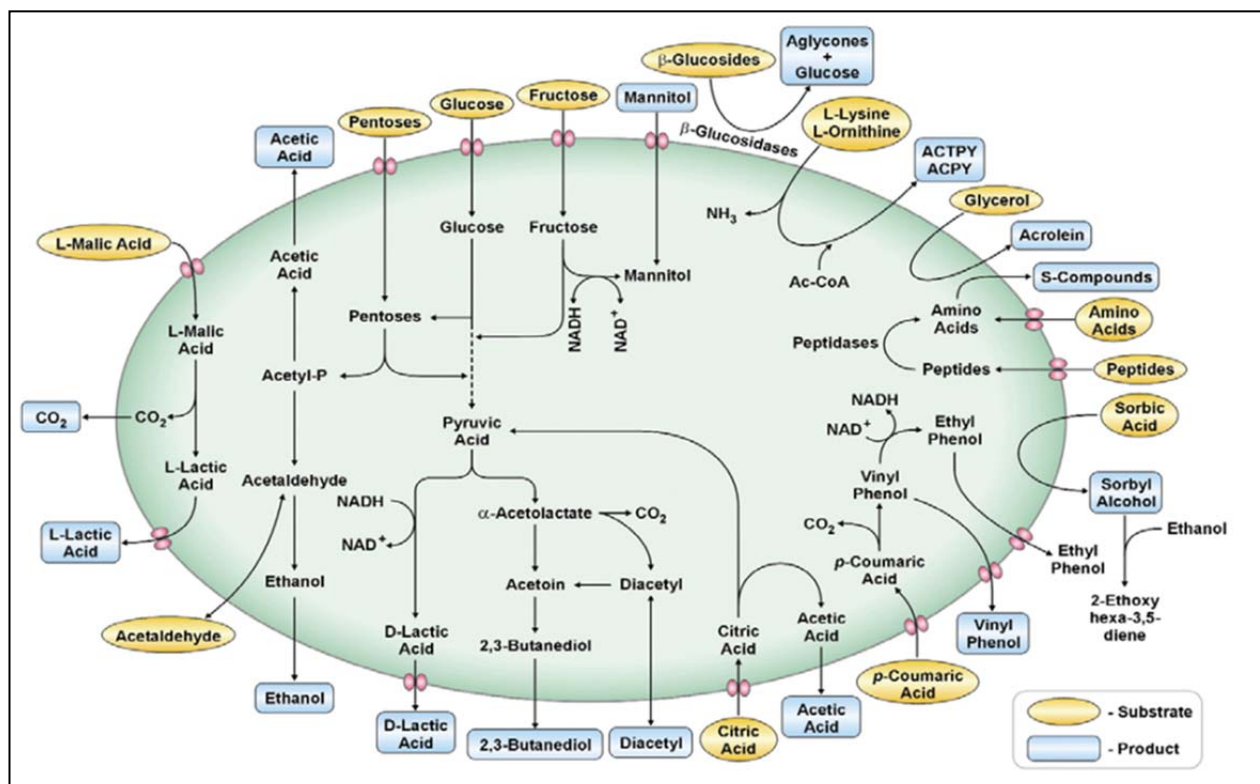


Figure 2.1 A schematic depiction of the biosynthesis and modulation of flavour-active compounds by malolactic bacteria (Swiegers *et al.*, 2005).

There are variations between strains in the production of volatile compounds, including ethyl and acetate esters, higher alcohols, carbonyl compounds, volatile fatty acids and sulphur compounds (Siebert *et al.*, 2005). Possible synergistic interactions between these volatile aroma compounds and constituents in the matrix may exist; this can also influence wine aroma (Ferreira *et al.*, 2000; Bartowsky and Borneman, 2011). A study by Pineau *et al.* (2009) found that the red- and blackberry aromas of red wines are made up of at least six different esters and volatile fatty acids.

Of all the aromatic groups that may be associated with MLF only esters, higher alcohols, volatile fatty acids, carbonyl compounds, monoterpenes and volatile sulphur compounds will be discussed.

2.5.1 Esters

Esters, generally associated with fruity aromas in wine, make up the major family (qualitatively and quantitatively) of volatile compounds released during yeast autolysis, occurring at the end of the stationary phase and associated with cell death (Alexandre and Guilloux-Benatier, 2006). Some studies have shown that the fermentation-derived ethyl- and acetate esters increase due to MLF (Maicas *et al.*, 1999, Ugliano and Moio, 2005), whilst others showed a decrease in esters (Gambaro *et al.*, 2001). Ethyl fatty acid esters are the product of the enzymatic esterification of activated fatty acids formed during lipid biosynthesis (Matthews *et al.*, 2004). Acetate esters form through the condensation of acetyl-CoA with higher alcohols (Ugliano and Henschke, 2008). The production of ethyl- and acetate esters is not necessarily similar during MLF. Ugliano and Moio (2005) found a greater increase in ethyl ester (such as ethyl acetate, ethyl lactate, ethyl hexanoate and ethyl octanoate) production than acetate ester production. It has also been mentioned that the concentration of some esters may decrease while others increase during storage of the wine and that this may be due to acid hydrolysis and chemical esterification (Liu, 2002).

Two important esters associated with MLF are ethyl lactate and diethyl succinate (Ugliano and Moio, 2005). Ethyl lactate is beneficial to the aroma profile due to its fruity, buttery and creamy aromas as well as its contribution to the mouthfeel of the wine (Ugliano *et al.*, 2003; Ugliano and Moio, 2005). Ethyl lactate is formed in the presence of lactic acid (produced by the LAB) and ethanol via esterification (Lerm, 2010). Diethyl succinate contributes fruity aromas to the wine (Peinado *et al.*, 2004) and is formed via the non-enzymatic esterification of succinic acid, a by-product of microbial α -ketoglutarate metabolism (Ugliano and Moio, 2005). The odour threshold of ethyl lactate and diethyl succinate is 110 mg/L and 1.2 mg/L, respectively (Lloret *et al.*, 2002; Peinado *et al.*, 2004). **Table 2.1** contains a list of some of the other esters associated with MLF and their possible contributions to wine aroma.

It has been shown that MLF causes an increase in esters, ethyl lactate and diethyl succinate in particular (Knoll *et al.*, 2011; Malherbe *et al.*, 2012). A study by Knoll *et al.* (2012) in Riesling showed that co-inoculated fermentations tended to have higher concentrations of ethyl- and acetate esters than sequentially inoculated fermentations and decreased concentrations of all acetate esters, except acetic acid, after MLF. In contrast to this, a study in Pinotage and Shiraz by Malherbe *et al.* (2012) observed a greater increase in ethyl esters than acetate esters due to sequentially inoculated MLF.

To summarize, the concentration of esters produced during MLF is dependent on LAB strains that exhibit different esterase activity. In general ester production is considered favourable due to the mostly fruity characters it imparts to wine aroma. Matthews *et al.* (2006) found that all LAB strains from the genera *Oenococcus*, *Lactobacillus* and *Pediococcus* that were screened, could hydrolyse esters. This signifies the use of LAB, from genera other than *Oenococcus*, to contribute to wine aroma.

Table 2.1 Odour quality, concentration and threshold of some esters found in wine, which contribute to the aroma during MLF (Lerm, 2010).

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

- not reported above threshold in any study

2.5.2 Higher alcohols

Higher alcohols are usually produced via the Ehrlich pathway from amino acids, which undergo transamination to form α -keto acids that are decarboxylated and subsequently reduced to form fusel alcohols (higher alcohols) (Hazelwood *et al.*, 2008). Higher alcohols can have either a positive or negative influence on wine aroma (Swiegers *et al.*, 2005). Optimal levels impart fruity aromas, whereas high concentrations can impart strong, pungent smell and taste (Swiegers and Pretorius, 2005; Swiegers *et al.*, 2005). If present in wine at concentrations below 300 mg/L, they add to the desirable complexity, but if present at concentrations exceeding 400 mg/L, they could influence the quality of the wine negatively (Swiegers and Pretorius, 2005).

Studies show contradictory results regarding the impact of MLF on higher alcohols. A study by Maicas *et al.* (1999) found an increase in total higher alcohols in wines that had undergone MLF and that the production of these higher alcohols is strain dependent. A study by Ugliano and Moio (2005) and Jeromel *et al.* (2008) found insignificant effects of MLF on higher alcohol production.

2.5.3 Volatile fatty acids

Volatile fatty acids are formed by the hydrolysis of lipids such as mono-, di- and triacylglycerols (Liu, 2002). In wine, the fatty acid content is comprised of straight chain- as well as branched chain fatty acids. The straight chain fatty acid content in wine is generally referred to as short chain (C_2 - C_4), medium chain (C_6 - C_{10}) or long chain (C_{12} - C_{18}) fatty acids (Ugliano and Henschke, 2008). The chain length of the fatty acids affects their volatility. An increase in chain length decreases their volatility and their odour changes from sour to rancid and cheese (Francis and

Newton, 2005; Ugliano and Henschke, 2008). The most commonly found volatile fatty acids in wine and their possible contributions are listed in **Table 2.2**.

Table 2.2 Odour quality, concentrations and odour thresholds of volatile fatty acids found in wine (Lerm, 2010).

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

- not reported above threshold in any study

Volatile fatty acids have been shown to be higher in wines that have undergone MLF (Maicas *et al.*, 1999; Herjavec *et al.*, 2001), but the increase in fatty acids with undesirable aromas could be detrimental to wine quality. Even though volatile fatty acids are usually present in wine in trace amounts, their low odour thresholds and pungent odours make them important contributors to wine aroma (Maicas *et al.*, 1999).

2.5.4 Carbonyl compounds

Diacetyl (2,3-butanedione) is a major secondary metabolite formed mainly via citric acid metabolism during MLF (Bartowsky *et al.*, 2002; Bartowsky and Henschke, 2004) (**Figure 2.2**).

Diacetyl imparts a buttery and butterscotch character (Bartowsky *et al.*, 2002; Swiegers *et al.*, 2005) that can add to wine complexity (Lerm, 2010). The odour detection threshold of diacetyl is cultivar dependent and its sensory perception depends on wine style, type, age, origin and the presence of other compounds in the wine (Swiegers *et al.*, 2005). Diacetyl concentrations exceeding 5 to 7 mg/L is regarded as undesirable, whereas concentrations between 1 and 4 mg/L contributes to the buttery and butterscotch aroma as well as add to wine complexity (Swiegers *et al.*, 2005; Lerm, 2010).

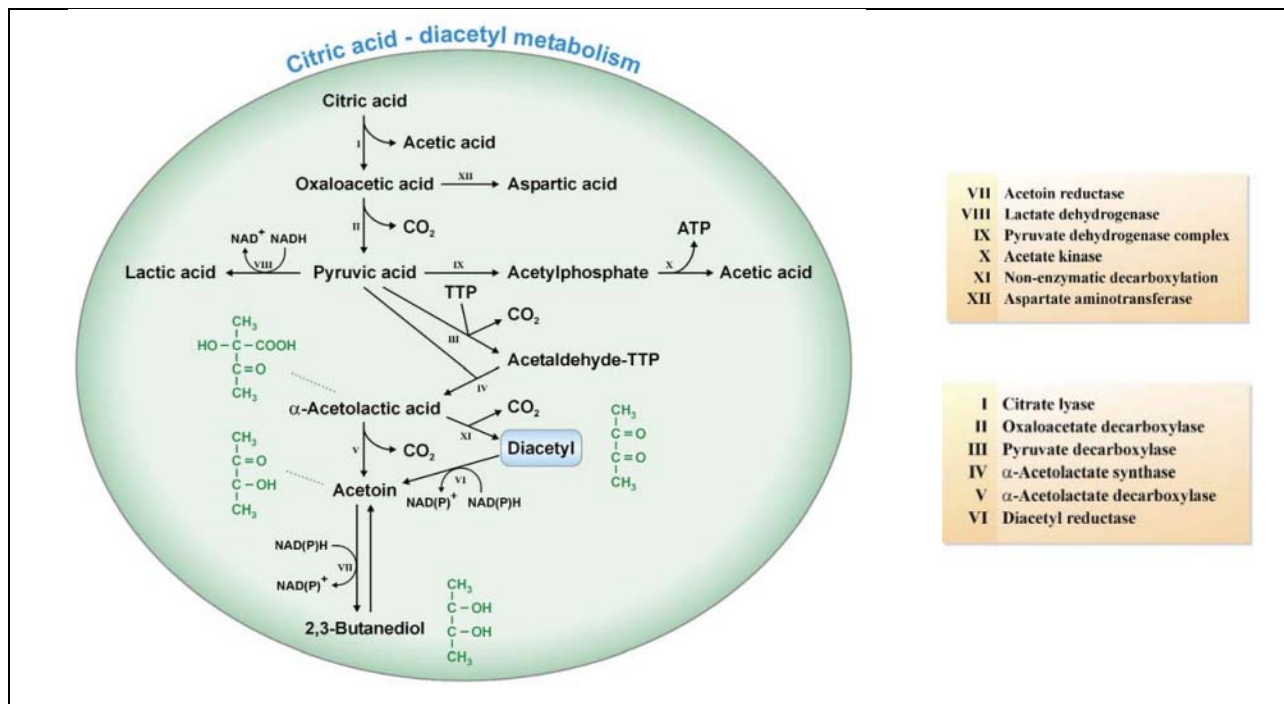


Figure 2.2 A schematic representation of citric acid metabolism and the diacetyl synthesis in LAB (Swiegers *et al.*, 2005).

Many factors can influence diacetyl production; these include bacterial strain, inoculation rate of the LAB, pH, citrate concentration, wine temperature, contact with lees following MLF, SO_2 concentration and degree of aeration during winemaking (Bartowsky *et al.*, 2002). For more detail see Bartowsky *et al.* (2002). Decreased pH values will increase microbially active SO_2 that will inhibit yeast and bacterial activity and therefore stabilise the diacetyl content of the wine.

As depicted in **Figure 2.2**, diacetyl is an intermediate metabolite that is unstable and can be further reduced to the less aroma active compounds acetoin and the alcohol, 2,3-butanediol (Bartowsky and Henschke, 2004). Acetoin and 2,3-butanediol are considered less aromatically significant due to their high aroma thresholds (approximately 150 and 600 mg/L, respectively) (Bartowsky and Henschke, 2004). Francis and Newton (2005) found that diacetyl levels were generally higher in aged red wines than young red wines. This might be due to the reversed action of SO_2 that bind to diacetyl during the initial addition of SO_2 after completion of MLF, resulting in an increase in diacetyl.

By managing the choice of LAB strain and other factors influencing diacetyl content, the winemaker can better manipulate the style of wine required.

2.5.5 Monoterpenes

The precursors of important monoterpenes (monoglucosides and diglucosides), such as linalool, geraniol, nerol, β -citronellol and α -terpineol, are synthesized during early grape berry development (Michlmayr *et al.*, 2012). Most of these monoterpenes are associated with pleasant aromas such as floral, rose-like and citrus (Swiegers and Pretorius, 2005; Michlmayr *et al.*, 2012).

Wine related LAB (*O. oeni*, *Lactobacillus* spp. and *Pediococcus* spp.) have the ability to hydrolyse various synthetic glycosides (Ugliano *et al.*, 2003; Grimaldi *et al.*, 2005a, b) thereby contributing to the attractive aroma profile during MLF. A study by Michlmayr *et al.* (2012) showed that glycosidases and arabinosidases from *O. oeni* can release high amounts of monoterpenes from natural substrates under optimal MLF conditions. Furthermore, a sensory panel preferred the enzyme-treated Riesling wines over the controls and confirmed that the glycosidases from *O. oeni* could improve the typical Riesling aroma.

Grimaldi *et al.* (2000) studied the influence of wine pH, ethanol and sugar concentrations on the β -glucosidase activity of *O. oeni* in synthetic medium and found that the presence of ethanol enhanced the β -glucosidase activity of *O. oeni*. A later study by Grimaldi *et al.* (2005b) in wine found that most of the *O. oeni* tested had high glycosidase activity at wine pH (between 3.0 and 4.0). A study by Michlmayr *et al.* (2012) found that at natural juice pH, the bacterial enzymes could still release significant amounts of terpenes, although at a lower magnitude.

2.5.6 Volatile sulphur compounds

Volatile sulphur compounds (VSC) play an important role in wine aroma due to their powerful and characteristic odours, although they are generally present at levels below or close to their detection thresholds, which adds to wine complexity (Du Toit *et al.*, 2011). Increased concentrations can impart undesirable odours that can be detrimental to wine quality (Lerm *et al.*, 2010). Examples of VSC found in wine and their possible aromatic contributions are listed in **Table 2.3**.

Table 2.3 The four main sulphur compounds produced by LAB during MLF and their possible impact on wine aroma (Landaud *et al.*, 2008)

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

For a long time, yeast metabolism was regarded as the sole contributor of VSC in wine until Pripis-Nicolau *et al.* (2004) proved that wine LAB can produce VSC from methionine during MLF. The four VSC they found were the same compounds listed in **Table 2.3**. The exact mechanisms and biochemical pathways responsible for VSC in LAB are still not completely understood (Du Toit *et al.*, 2011). Vallet *et al.* (2008) proposed a possible pathway by which VSC is formed by LAB. They proposed that methionine metabolism by LAB leads to the formation of methanethiol, dimethyl disulphide, 3-(methylsulphanyl)propan-1-ol (aka methionol) and 3-(methylsulphanyl)propionic acid.

In the study done by Pripis-Nicolau *et al.* (2004), they found that, of the four main VSC, 3-(methylsulphanyl)propionic acid was the only compound that showed significantly higher levels in Merlot. The same study found that *O. oeni* produced higher VSC levels than the other *Lactobacillus* spp. they tested, leading to the conclusion that VSC production by LAB is genera as well as strain dependent. A study by Ugliano and Moio (2005) found increased levels of methionol following MLF and that the concentrations produced varied according to grape variety, probably relating to the availability of precursors. The chemical matrix also seems to impact the aroma perception of VSC. Pripis-Nicolau *et al.* (2004) found that in synthetic medium, 3-(methylsulphanyl)propionic acid was described as having chocolate or roasted aromas and an odour threshold value of 50 µg/L. In wine the same compound was described as having earthy and red fruit aromas and an odour threshold value of 244 µg/L.

Further investigation is needed relating to the production of VSC by LAB and the associated mechanisms and biochemical pathways. The two factors that seem to influence VSC the most during MLF are both factors that the winemaker can manipulate; namely, LAB strain and grape variety.

2.6 Concluding remarks

Knowledge of MLF, LAB and the factors that influence them assist winemakers to ensure that MLF will complete successfully, which entails the successful degradation of malic acid, leaving a microbiologically sound wine with the desired aroma and without undesired aromas or off-flavours.

The physiochemical factors that can influence MLF, which winemakers can control, include temperature, SO₂ additions and pH. By maintaining temperatures between 18 and 22°C, a total SO₂ concentration of below 30 mg/L and ensuring a pH of 3.2 to 3.4 will optimize the conditions for the survival and growth of *O. oeni* (Lerm, 2010). If *L. plantarum* is used in the starter culture, then a wine pH of higher than 3.5 needs to be maintained. The choice of yeast strain to perform AF and bacterial strain/s to perform MLF is another aspect the winemaker can control that will influence MLF. Antagonistic interactions can be minimized by selecting compatible yeast strains for MLF. The yeast strain should produce low amounts of inhibitory metabolites such as SO₂ and medium chain fatty acids that may inhibit LAB growth.

By inoculating with a commercial starter culture, the risks associated with natural or uninoculated (spontaneous) MLF is reduced. Commercial starter cultures may contain *O. oeni*, *L. plantarum* or a mixture of the two. The choice of starter culture will mainly depend on the desired style of wine. These commercial starter cultures are selected for their tolerance to the harsh wine environment to successfully conduct MLF and their impact on wine quality, especially aroma. By inoculating with a mixed culture of *O. oeni* and *L. plantarum*, the winemaker ensures that, within reasonable limits, at any wine pH, at least one of the inoculated LAB will dominate MLF. By using co-inoculation, MLF duration can be shortened; risks associated with sequential inoculation reduced and contribute to the desired aroma profile of the wine.

Lactic acid bacteria can produce a range of aroma compounds that may contribute negatively or positively to the wine and this production is dependent on genera as well as strain. It has been proven by many studies that MLF, especially inoculated MLF, can improve wine aroma. Future studies of the factors influencing these aroma compounds will provide winemakers to better manage a specific type and style of wine. Different LAB genera and strains as well as mixtures of LAB cultures should be considered for starter culture use.

It is important for winemakers to monitor MLF on a continuous basis. Winemakers should keep a keen eye on malic acid degradation and the LAB responsible for fermentation. This will enable winemakers to better manage MLF in case of problem fermentations that may impact wine quality, especially aroma.

The amount of studies associated with MLF is increasing and leading to a better knowledge of the complex process that may cause concern for many winemakers. Better knowledge of this process will provide winemakers with the appropriate knowledge to optimally manage MLF with informed choices to improve their specific style of wine.

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Chapter 3

Research results

**Impact of yeast and nutrient addition on the NT 202
Co-Inoculant mixed MLF starter culture and the
aroma compound production in the final wine**

3. Research results

Impact of yeast and nutrient addition on the NT 202 Co-Inoculant mixed MLF starter culture and the aroma compound production in the final wine

3.1 Introduction

Malolactic fermentation (MLF) is a secondary fermentation process that occurs in most red and some white wines usually after the completion of alcoholic fermentation (AF) or concurrently with AF. Malolactic fermentation is a decarboxylation process where the harsher tasting L-malic acid is converted into the softer tasting L-lactic acid (Abrahamse and Bartowsky, 2012). This conversion is catalysed by the malolactic enzyme (mle) produced by lactic acid bacteria (LAB) such as *Oenococcus oeni* and *Lactobacillus plantarum* present in the grape must or wine. Malolactic fermentation is important during vinification because it not only deacidifies the wine, but it also improves microbial stability and wine flavour (Du Toit *et al.*, 2011; Abrahamse and Bartowsky, 2012).

The most important LAB indigenous to the wine environment belong to the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Lerm *et al.*, 2010). Commercial starter cultures are usually comprised of *O. oeni* strains, but recently the use of *L. plantarum* in commercial starter cultures has shown a significant contribution as these LAB are able to survive the harsh wine environment, which includes high ethanol concentrations, pH, temperatures and sulphur dioxide (Du Toit *et al.*, 2011).

The recent development of a mixed MLF starter culture consisting of *O. oeni* and *L. plantarum*, developed to be used in co-inoculation, proves that the implementation of co-inoculation is increasing in the industry. Not only does co-inoculation provide the potential for more efficient tank usage in the cellar due to shortened fermentations, but it also improves the wine aroma and serves as a tool to help the LAB overcome high ethanol concentrations associated with e.g. sequential inoculation (Jussier *et al.*, 2006; Lerm *et al.*, 2010).

Microorganisms, present in the grape must or wine, form a diverse microbiological ecology and not all microorganisms are favourable. These microorganisms include fungi, yeasts and bacteria that can interact with each other and influence one another's survival (Fleet, 2003; Alexandre *et al.*, 2004). Factors that can inhibit LAB growth includes: high ethanol- and sulphur dioxide (SO₂) concentrations, medium chain fatty acids, high pH, extreme temperatures and some phenolic acids (Reguant *et al.*, 2000; Lerm *et al.*, 2010). For co-inoculation as a MLF inoculation regime, it is important that the selected yeast strain does not inhibit or influence the selected LAB strain/s or wine quality in a negative way. Apart from alcohol production by yeast during AF (which can inhibit LAB growth) studies have showed that MLF can be inhibited or negatively influenced by peptides produced by yeasts

during AF (Osborne and Edwards, 2007; Nehme *et al.*, 2010). Yeast-synthesized medium chain fatty acids such as hexanoic, octanoic, decanoic and dodecanoic acids can inhibit bacterial growth and MLF (Carreté *et al.*, 2002; Lerm, 2010).

Various additions to wine have become standard practice in many wineries today. These additions include yeast- and bacterial nutrients, detoxifying- and clarification agents. The nutritional additives that contain inactivated yeasts can provide organic nitrogen, available amino nitrogen, phosphates, cell wall polysaccharides, cellulose, mineral cofactors as well as vitamins. Lactic acid bacteria are affected by mannoprotein additions. Yeast commercial mannoproteins of intermediate molecular weight (6-22kD) have been proven to increase *O. oeni* growth in the presence of ethanol (Diez *et al.*, 2010). To detoxify wines, yeast hulls (ghost yeasts) can be added and will serve a double purpose by providing bacterial nutrients and aiding in successful MLF by reducing antagonism by growing yeasts (Du Toit *et al.*, 2011).

Wine flavour is influenced positively and negatively by the microbiological profile of a wine (Fleet 2003). It has been established that yeast influence wine aroma, but LAB also contribute to the final sensory properties of wine (Swiegers *et al.*, 2005). These aromatic contributions of the LAB to the wine are also strain dependant (Bartowsky and Borneman, 2011). The effect of MLF on wine aroma usually include final wine descriptors such as buttery and nutty, whereas co-inoculation leads to more fruity, less buttery wines (Lerm *et al.*, 2010). The *L. plantarum* species possess β -glucosidase activity that can modify the sensorial profile of the wine by hydrolysing sugar-bound monoterpenes to release the volatile, aromatic monoterpenes (Liu, 2002; Michlmayr *et al.*, 2010; Mtshali *et al.*, 2010).

In this study, the focus will be on 1) assessing the impact of different white and red wine yeast strains on the ability of the NT 202 Co-Inoculant to conduct MLF, 2) assessing the impact of the yeast-LAB combinations on the aroma compounds produced in the final wine and 3) assessing the impact of commercial additives such as nutrients, detoxifying and clarifying agents on MLF kinetics and aroma compound production, when used in co-inoculation.

3.2 Materials and methods

3.2.1 Vinification procedures

In the 2011 vintage Chardonnay and Merlot grapes were used to conduct small-scale vinifications and for the 2012 vintage Shiraz grapes were used. The Chardonnay and Merlot grapes were sourced from the Elgin region, Western Cape, South Africa, and the Shiraz grapes were sourced from the Stellenbosch region, Western Cape, South Africa. Treatments were done in triplicate for the Chardonnay and Shiraz and in duplicate for the Merlot. This was due to the number of fermentations (86) to be handled at once. In 2011 the fermentations were used as screening of yeast strains to be tested in 2012. Standard vinification procedures were followed. Co-inoculation was used for both vintages. The red wine fermentations were conducted at 25°C and the Chardonnay fermentations at 15°C.

Half a ton of Chardonnay and Shiraz and a ton of Merlot were crushed and destemmed. The Chardonnay fermentations were conducted in 4.5 L glass bottles (sealed with airtight fermentation caps) and the Merlot in 10 kg plastic buckets. Representative samples of the respective cultivars were taken and standard wine parameters (sugar, pH, TA, free SO₂ and total SO₂) were measured. Sulphur dioxide was added to must to achieve a total SO₂ concentration of 20 ppm prior to onset of AF to suppress indigenous microflora growth in the 2011 vintage. No SO₂ adjustments were made prior to AF in the 2012 vintage due to an already high SO₂ concentration present on the Shiraz grapes. The Chardonnay grapes were crushed, destemmed and then pressed in a hydraulic press prior to 24 hours settling at 4°C. After settling, the Chardonnay must was homogeneously divided into 4.5 L aliquots for fermentation. For both red cultivars the grapes were crushed, destemmed and then divided into roughly 9 kg aliquots for fermentation. After completion of MLF, the wines (except for Chardonnay) were pressed in a hydraulic press, racked, SO₂ added to achieve a total SO₂ concentration of 70 ppm and then cold stabilized at 4°C prior to bottling.

3.2.2 Treatments

For the 2011 vintage, 14 different yeast strains were evaluated in Merlot (**Table 3.1**) and four different yeast strains in Chardonnay (**Table 3.2**). For the 2012 vintage four yeast strains were selected from the 2011 vintage and evaluated in Shiraz (**Table 3.3**). Each of the LAB MLF starter cultures were co-inoculated with the various yeast strains listed in **Tables 3.1** to **3.3**. A yeast strain was selected per cultivar to conduct treatment controls in duplicate for the 2011 vintage and in triplicate for the 2012 vintage. The *S. cerevisiae*/*Saccharomyces paradoxus* hybrid Exotics (Anchor Wine Yeast) was used as the control yeast strain for Chardonnay and *S. cerevisiae* NT 202 (Anchor Wine Yeast) as control for Merlot and Shiraz. Nutrivin (Anchor Wine Yeast) was added as yeast nutrition at 0.7 g/L on the

second day of fermentation. No nutrition was added for the bacteria. Also in the 2012 vintage, seven different additives were evaluated in Shiraz using yeast strain *S. cerevisiae* NT 202 and MLF starter culture NT 202 Co-Inoculant in co-inoculation (**Table 3.4**). Manufacturer's specifications were followed for all inoculation practices. Manual punch downs were done daily in red wine fermentations in order to ensure sufficient skin contact. Sugar concentrations were monitored daily by Fourier Transform Mid-Infrared Spectroscopy (FT-MIR) using the WineScan FT120 (**section 3.2.5**).

Table 3.1 Yeast- and bacterial strains used in the Merlot 2011. Treatments were evaluated in duplicate and treatment abbreviations for the MLF starter cultures are referred to in brackets.

MLF starter cultures	Yeast starter cultures
NT 202 Co-Inoculant (Anchor Wine Yeast) (MIX)	<i>S. cerevisiae</i> NT 202
Viniflora oenos (Chr. Hansen) (VO)	Lalvin EC-1118 (Lallemand) (EC1118)
Lalvin VP 41 (Lallemand) (VP41)	Fermivin (DSM)
	Fermirouge (DSM)
	Fermicru VR5 (DSM)
	Fermicru XL (DSM)
	Collection Cépage Merlot (DSM) (CC Merlot)
	Collection Cépage Cabernet (DSM) (CC Cabernet)
	Collection Cépage Pinot (DSM) (CC Pinot)
	Exotics SPH (Exotics)
	<i>S. cerevisiae</i> NT 50 (Anchor Wine Yeast)
	<i>S. cerevisiae</i> NT 112 (Anchor Wine Yeast)
	<i>S. cerevisiae</i> WE 372 (Anchor Wine Yeast)
	<i>S. cerevisiae</i> NT 116 (Anchor Wine Yeast)
Control: no bacterial inoculation	Control: NT 202

Table 3.2 Yeast- and bacterial strains used in the Chardonnay 2011. Treatments were evaluated in triplicate.

MLF starter cultures	Yeast starter cultures
NT 202 Co-Inoculant	VIN 2000 (Anchor Wine Yeast)
Viniflora CH35	VIN 13 (Anchor Wine Yeast)
Lalvin VP41	Fermicru LVCB (DSM)
	Exotics SPH
Control: No bacterial inoculation	Control: Exotics

Table 3.3 Yeast- and bacterial strains used in the Shiraz 2012. Treatments were evaluated in triplicate.

MLF starter cultures	Yeast starter cultures
NT 202 Co-Inoculant	Exotics SPH
Lalvin VP41	NT 50
	WE 372
	NT 202
Control: No bacterial inoculation	

Table 3.4 Additives evaluated in Shiraz in 2012. Treatments were evaluated in triplicate.

Additives	Manufacturer
Extraferm	DSM
Natuferm	Oenobrand
Claristar	DSM
OptiMalo Plus	Lallemand
Bactiv-Aid	Chr. Hansen
Nutrivin	Anchor Wine Yeast
Control (Predferm D.A.P)	Brenn-O-Kem, Prédél Oenologie

3.2.3 Sampling

Wines were punched down and mixed before sampling was done to ensure a homogenous matrix. Representative grape must and/or wine samples were then taken for analysis of standard parameters and microbial enumeration.

Chardonnay 2011

Regarding the standard analysis, sampling took place daily for the first three weeks and weekly thereafter. For LAB enumeration, sampling took place every third day of MLF and for *S. cerevisiae* enumeration; sampling took place at onset, middle and end of AF.

Merlot 2011

Due to the experimental layout of the 2011 Merlot and practical feasibility, sampling was divided into two groups that were sampled every second day for microbiological enumeration and standard wine analysis until completion of MLF.

Shiraz 2012

Sampling for standard wine analysis took place every day for the first week then at two or three day intervals until completion of MLF. Sampling for microbial enumeration for *S. cerevisiae* and LAB were done every second or third day until completion of AF, followed by sampling two and then three days thereafter for LAB enumeration until completion of MLF.

3.2.4 Microbial enumeration

For microbial enumeration, representative samples of the grape must were drawn before inoculation to determine the indigenous yeast and LAB flora present. To monitor the microbiological status of the

fermenting grape must, samples were plated out on the following media : Yeast Peptone Dextrose (YPD) agar (Biolab, Merck, South Africa) (70 g/L), De Man, Rogosa and Sharpe (MRS) agar as well as MRS, supplemented with 10% tomato juice (MRST) agar (**Table 3.5**). Hydrochloric acid (Merck, South Africa) was used to adjust the pH of the MRST agar to pH 5.

Table 3.5 Ingredients used for MRS and MRST media.

	MRS agar medium	MRST agar medium
MRS broth (Biolab, Merck, South Africa)	50 g/L	50 g/L
Agar bacteriological (Biolab, Merck, South Africa)	15 g/L	20 g/L
Tomato juice, preservative free (All Gold, South Africa)	-	100 mL/L

Antibiotics were added to all media to inhibit the growth of certain microorganisms. Kanamycin sulphate (Roche Diagnostics GmbH, Mannheim, Germany) was added at 25 mg/L to all media to inhibit the growth of acetic acid bacteria. Chloramphenicol (Roche Diagnostics GmbH, Mannheim, Germany) was added at 50 mg/L (dissolved in 1 mL of 96% ethanol) to the YPD agar to inhibit the growth of LAB. Delvocid Instant (DSM Food Specialities, The Netherlands) was added at 50 mg/L (dissolved in 1 mL of sterile, de-ionised water) to the MRS- and MRST agar to inhibit the growth of yeasts and moulds.

Ten-fold dilution series of the grape must or wine in sterile de-ionised water were made and 100 μ L was plated out on the respective media. The plates were incubated at 30°C for three to ten days depending on the microbial growth. The plate counts were monitored as colony forming units per millilitre (cfu/mL). Light microscopy was used to do spot checks on the counted colonies to investigate cell morphologies.

3.2.5 Standard analyses

A WineScan FT120 spectrophotometer (FOSS Analytical, Denmark) was used to perform the analysis of the majority of the standard parameters of the juice and wine utilising FT-MIR to generate spectra in the wavenumber region 929-4992 cm^{-1} as described by Malherbe (2010).

The standard juice parameters that were analysed include: reducing sugar, ethanol, pH, tartaric- and volatile acid concentration, glucose and fructose concentrations as well as malic acid and lactic acid concentrations. The standard wine analysis include: pH, malic- and lactic acid, total and volatile acidity, glucose and fructose and ethanol. Total- and free SO_2 analysis were done using the Metrohm titration unit (Metrohm Ltd., Switzerland). Enzyme kits were used in conjunction with the Arena 20XT (Thermo Electron Oy, Finland) according to manufacturer's instructions to determine the malic acid (Enzytec™ Fluid L-Malic acid Id-No: 5280. Thermo Fisher Scientific Oy, Finland distributed by R-

Biopharm AG, Germany) and lactic acid (Enzytec™ Fluid L-Lalic acid Id-No: 5260. Thermo Fisher Scientific Oy, Finland distributed by R-Biopharm AG, Germany) concentrations.

The purpose of these standard parameter analyses was to routinely monitor the chemical compounds present in the wine and to generate spectra to be used for data analysis (**section 3.2.6**). The acquisition and processing of the spectral data took place as described by Nieuwoudt *et al.* (2004).

3.2.6 Volatile aroma compounds

Aroma compound analyses were done for the 2011 Merlot and 2012 Shiraz after completion of MLF using gas chromatography (GC). The major volatiles (esters, higher alcohols and volatile fatty acids) as well as the monoterpenes were measured using gas chromatography – flame ionized detection (GC-FID) whereas the carbonyl compounds were quantified using GC-mass spectrometry (GC-MS), coupled with solid-phase microextraction (SPME).

Major volatiles

The major volatiles were extracted as previously described by Louw *et al.* (2009) with the following exception: centrifugation of the wine/ether mixture at 4000 g for only three minutes, after which Na₂SO₄ was added to the mixture and the centrifugation step, was repeated.

A Hewlett Packard 6890 Plus gas chromatograph (Agilent, Little Falls, Wilmington, USA) was used to analyse for the major volatile aroma compounds (**Table 3.6**). The GC was equipped with a split/splitless injector, set to a split flow rate of 98.7 mL/min, split ratio of 15:1 and a temperature of 200°C. Separation of the compounds were done using a J & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions of 60 m length x 0.32 mm internal diameter with a 0.5 µm coating film thickness and using a hydrogen carrier gas flow rate of 6.6 mL/min. An injection volume of 3 µL of the extracted sample was used. The oven temperature program was as follows: 33°C, held for 8 minutes, increased by 21°C/min to 130°C, held for 1.3 min, then increased by 21°C/min to 170°C. The oven temperature was held at 170°C for 1 min and finally increased by 21°C/min to 240°C and then held for 2.5 min. The FID was operated at 250°C with a hydrogen flow of 30 mL/min, oxygen at 350 mL/min and make-up gas flow of nitrogen at 30 mL/min. A post run, between runs, was done for 5 min at 240°C. Thermal and chemical cleaning of the column was done by hexane injection after approximately every 24 samples, with a holding time of 10 minutes per injection. Calibration for each of the compounds was done using the internal standard method and authentic standards (Merck, Cape Town). Manual data collection and peak integration was performed using the HP Chemstation software (Rev. B01.03 [204]).

Monoterpenes

The method for extraction and quantification of the monoterpenes were done as described by Zietsman *et al.* (2011). A list of the monoterpenes quantified with GC-FID can be seen in **Table 3.6**.

Carbonyl compounds

Samples were collected from the red wines produced in the 2011 and 2012 vintages and the principal carbonyl compounds (diacetyl, 2,3-pentadione and acetoin) extracted and quantified as described by Malherbe *et al.* (2012).

3.2.7 Data analyses

Multivariate data analysis techniques, including principal component analysis (PCA) was used for statistical analysis using Statistica version 10 (Statsoft Inc.) and *The Unscrambler* software (version 9.2, Camo ASA, Norway).

Table 3.6 The major volatile aroma compounds and monoterpenes quantified by GC-FID analysis in the 2011 Merlot and 2012 Shiraz.

Major volatiles			Monoterpenes
Esters	Fatty acids	Higher alcohols	
Ethyl Acetate	Acetic Acid	Methanol	Limonene
Ethyl Propionate	Propionic Acid	Propanol	Fenchone
Ethyl-2-methylpropanoate	Isobutyric Acid	Isobutanol	Linalooloxide 1
2-Methyl-propyl Acetate	Butyric Acid	Butanol	Linalooloxide 2
Ethyl Butyrate	Isovaleric Acid	Isoamyl Alcohol	± Linalool
Ethyl-2-methylbutyrate	Valeric Acid	Pentanol	Linalyl Acetate
Ethyl Isovalerate	Hexanoic Acid	4-Methyl-1-pentanol	α-Terpeneol
Isoamyl Acetate	Octanoic Acid	3-Methyl-1-pentanol	Citronellol
Ethyl Hexanoate	Decanoic Acid	Hexanol	Nerol
Hexyl Acetate		3-Ethoxy-1-propanol	Geraniol
Ethyl Lactate		1-Octen-3-ol	α-Ionone
Ethyl Caprylate		2-Phenylethanol	β-Ionone
Ethyl-3-hydroxybutanoate			β-Farnesol 1
Ethyl Caprate			β-Farnesol 2
Diethyl Succinate			β-Farnesol 3
Ethyl Phenylacetate			
2-Phenylethyl Acetate			

3.3 Results and discussion

3.3.1 Fermentation kinetics

The chemical composition of the grapes from the Elgin and Stellenbosch regions that were used in the 2011 and 2012 vintages for co-inoculation are listed in **Table 3.7**.

The must malic acid concentrations varied, with the highest concentration of 4.1 g/L present in the Chardonnay of 2011 and the lowest of 1.12 g/L in the Merlot of 2011.

Table 3.7 Chemical composition of the grape musts used for vinifications during the 2011 and 2012 vintages.

Grape variety	Growing region	Vintage	pH	Total acidity (g/L)	Sugar concentration (°B)	Malic acid (g/L)
Chardonnay	Elgin	2011	3.36	9.32	20.9	4.10
Merlot	Elgin	2011	3.61	4.32	25.0	1.12
Shiraz	Stellenbosch	2012	3.82	3.74	25.5	1.24

Chardonnay 2011

The 2011 Chardonnay was harvested at 20.9°B, instead of the expected 24°B, and therefore the sugar concentration of the crushed grapes were adjusted to 24°B with cane sugar (Huletts, South Africa) to simulate a warmer climate region Baling. The AF for all evaluated yeast strains finished within 26 days, except Exotics and the un-inoculated MLF control, which took more than 40 days to complete (data not shown). The MLF stuck for the majority of the yeast treatments for NT 202 Co-Inoculant and the fermentations were discontinued after six weeks due to lack of malic acid degradation (**Figure 3.1**). The reason for the stuck MLF is probably due to the inadequate adaptation of the LAB to the harsh wine environment caused especially by the high acidity, low pH and high malic acid concentration (**Table 3.7**). The stuck MLF trends are also visible in the total LAB counts (**Figure 3.9**).

According to the malic acid degradation of the yeast/LAB combinations used in co-inoculation, Exotics showed the most promising results (**Figure 3.1**). Exotics, co-inoculated with Viniflora CH35 and Lalvin VP41 both containing just *O. oeni*, were the only treatments that completed MLF (malic acid concentration less than or equal to 0.3 g/L) in six weeks. This is in contrast with the co-inoculated yeast treatments VIN 2000, VIN 13, Fermicru LVCB and the un-inoculated MLF control that did not degrade malic acid to concentrations below 0.3 g/L in a six week time period. Exotics (co-inoculated with NT 202 Co-Inoculant) degraded malic acid down to 2 g/L. This malic acid degradation shown by yeast treatment Exotics, regardless of the MLF starter culture used might be due to the ability of the *S. paradoxus* yeast strain to degrade malic acid as shown by a study by Redzepovic *et al.* (2003) using Chardonnay. The *O. oeni* starter cultures, Viniflora CH35 and Lalvin VP41, showed similar malic acid degradation compared to the mixed MLF starter culture NT 202 Co-Inoculant. This indicates that the

presence of the *L. plantarum* in the mixed starter culture do not affect the malic acid degradation rate compared to the *O. oeni* cultures.

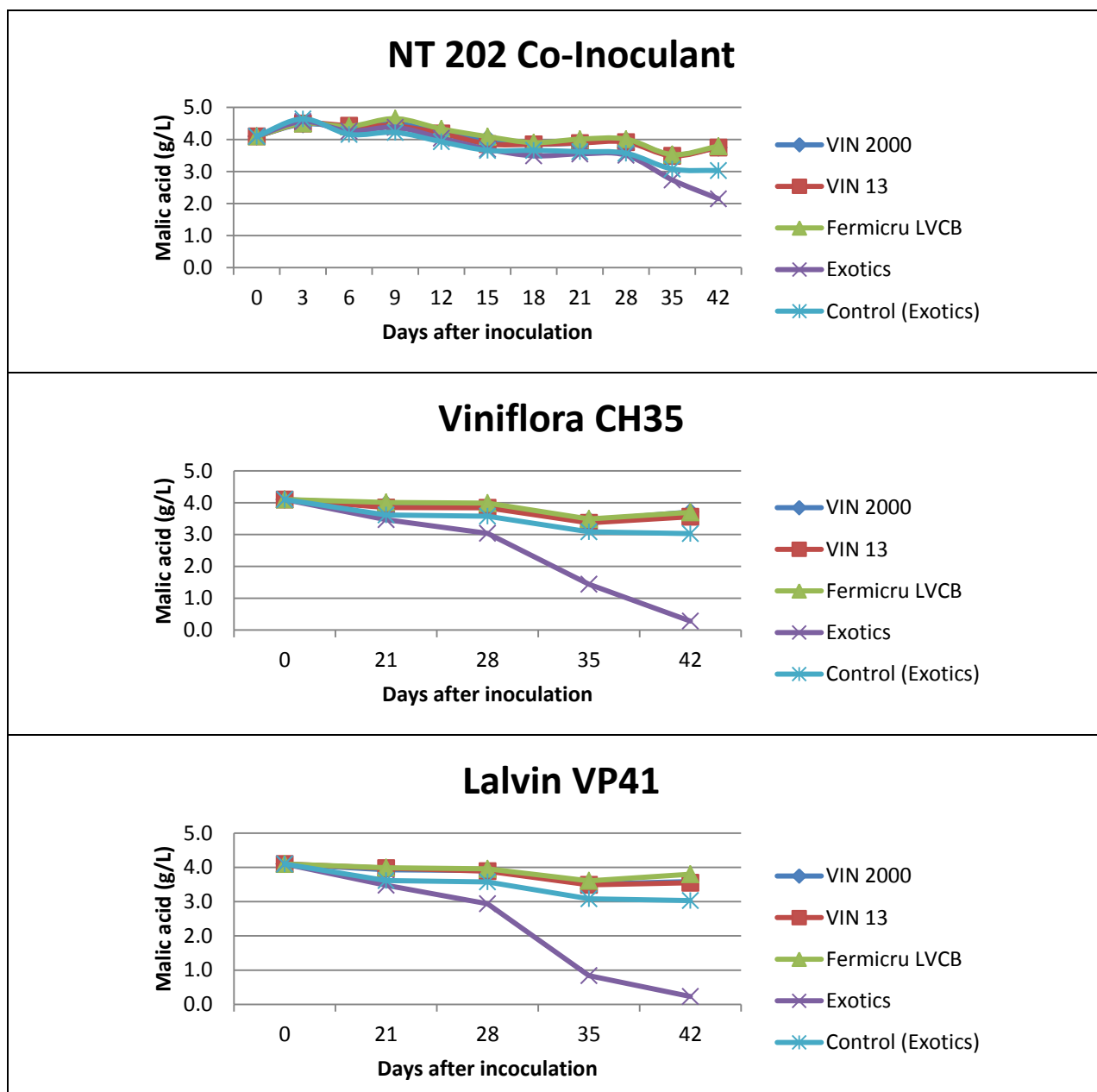


Figure 3.1 Malic acid degradation graphs of the NT 202 Co-Inoculant, Viniflora CH35 and Lalvin VP41, co-inoculated with the different yeast strains, observed in Chardonnay 2011 vintage. Data shown represents average malic acid (g/L) values of triplicate treatments.

Merlot 2011

The AF and MLF were successful for the majority of the yeast treatments. Alcoholic fermentation finished within 13 days and MLF finished within eight days for the majority of the treatments (**Figs 3.2 to 3.5**). According to the malic acid degradation abilities of the three different MLF starter cultures tested in co-inoculation with the 14 yeast strains, the yeast, when co-inoculated with NT 202 Co-Inoculant, could be grouped into three distinct categories: inhibitory towards MLF (displaying a longer lag phase) (**Figure 3.2**), neutral towards MLF (**Figure 3.3**) or stimulatory towards MLF (**Figure 3.4**). This type classification was used by Patynowsky *et al.* (2002) to designate yeast strains as being inhibitory, neutral or stimulatory to the growth of *O. oeni*. Even the yeast strains grouped under the 'inhibitory towards MLF' category allowed the completion of MLF in seven to eight days. This indicated that despite the impact of different yeast strains on the bacterial starter culture, MLF success was not affected for the majority of the yeast strains tested. Fermicru VR5 and Fermicru XL were the only two yeast strains that did not complete MLF in nine days like the rest of the yeast treatments (**Figure 3.5**). There were almost no differences observed in the total length of MLF between the three categories (**Table 3.8**).

The yeast treatments NT 202, Fermivin, CC Merlot and Fermirouge were classified as inhibitory towards MLF due to their delayed degradation of malic acid in the initial phase compared to the uninoculated control and the pure *O. oeni* cultures (Viniflora oenos and VP41) (**Figure 3.2**). In all cases Viniflora oenos performed the best in co-inoculation, followed by VP41 and NT 202 Co-Inoculant in terms of malic acid degradation. When co-inoculated with Viniflora oenos, yeast treatments NT 202 and CC Merlot completed MLF in four days and yeast strains Fermivin and Fermirouge completed MLF in five days. VP41 and NT 202 Co-Inoculant allowed MLF to be completed in seven to eight days when co-inoculated with NT 202, Fermivin, CC Merlot and Fermirouge. This delay in malic acid degradation could be the result of interactions between the specific yeast strain and the *L. plantarum* present in the NT 202 Co-Inoculant starter culture (Lerm, 2010). Lactic acid bacteria also vary in their tolerance to inhibitory conditions. It is known that *O. oeni* is better adapted to harsh wine conditions than *L. plantarum* (Lonvaud-Funel, 1999). This could explain the delay in its adaptation to the wine after inoculation and therefore the delay in malic acid degradation, independently of the yeast strain. In general these results show that yeast- and bacterial strain have an impact on MLF (Nehme *et al.*, 2008).

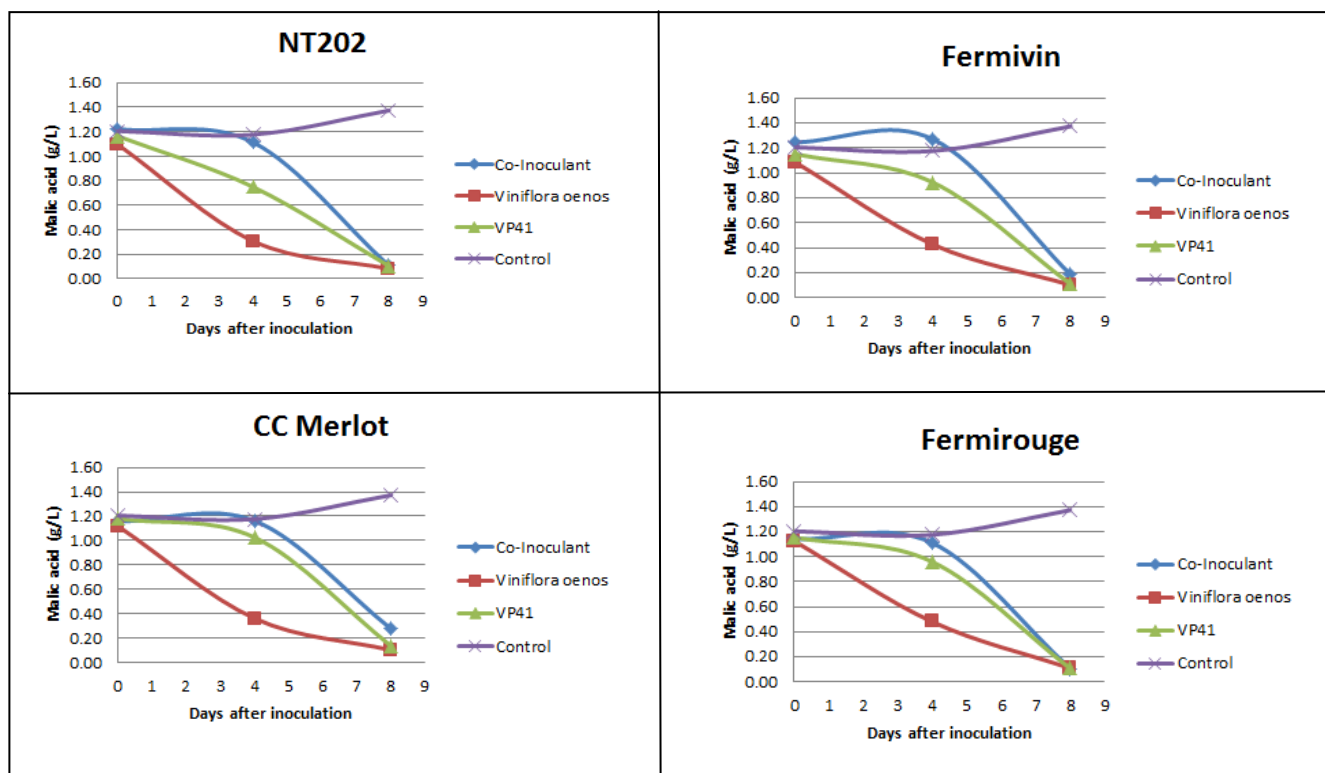


Figure 3.2 Malic acid degradation graphs of the different MLF starter cultures per yeast, in co-inoculation, observed in Merlot 2011 vintage. Data shown are averaged values of duplicate treatments (standard deviations not shown).

Yeasts CC Cabernet, NT112, NT50 and EC1118 were classified as neutral towards MLF due to their rate of malic acid degradation compared to the un-inoculated control and the pure *O. oeni* cultures (Viniflora oenos and VP41) (**Figure 3.3**). The performance of the Viniflora oenos and the VP41, in terms of malic acid degradation, could not be distinguished as clearly seen in **Figure 3.2**, although EC1118 did show a slightly faster degradation of malic acid when co-inoculated with Viniflora oenos than with VP41 (**Figure 3.3**).

Yeast strains CC Pinot, Exotics, WE372 and NT116 were classified as stimulatory towards MLF due to their malic acid degradation rates when compared to the un-inoculated control and the pure *O. oeni* cultures (Viniflora oenos and VP41) (**Figure 3.4**). In terms of malic acid degradation, NT 202 Co-Inoculant performed very similar to Viniflora oenos and VP41. When co-inoculated with CC Pinot and Exotics, MLF completed in six to seven days and WE372 and NT116 completed MLF in five to six days.

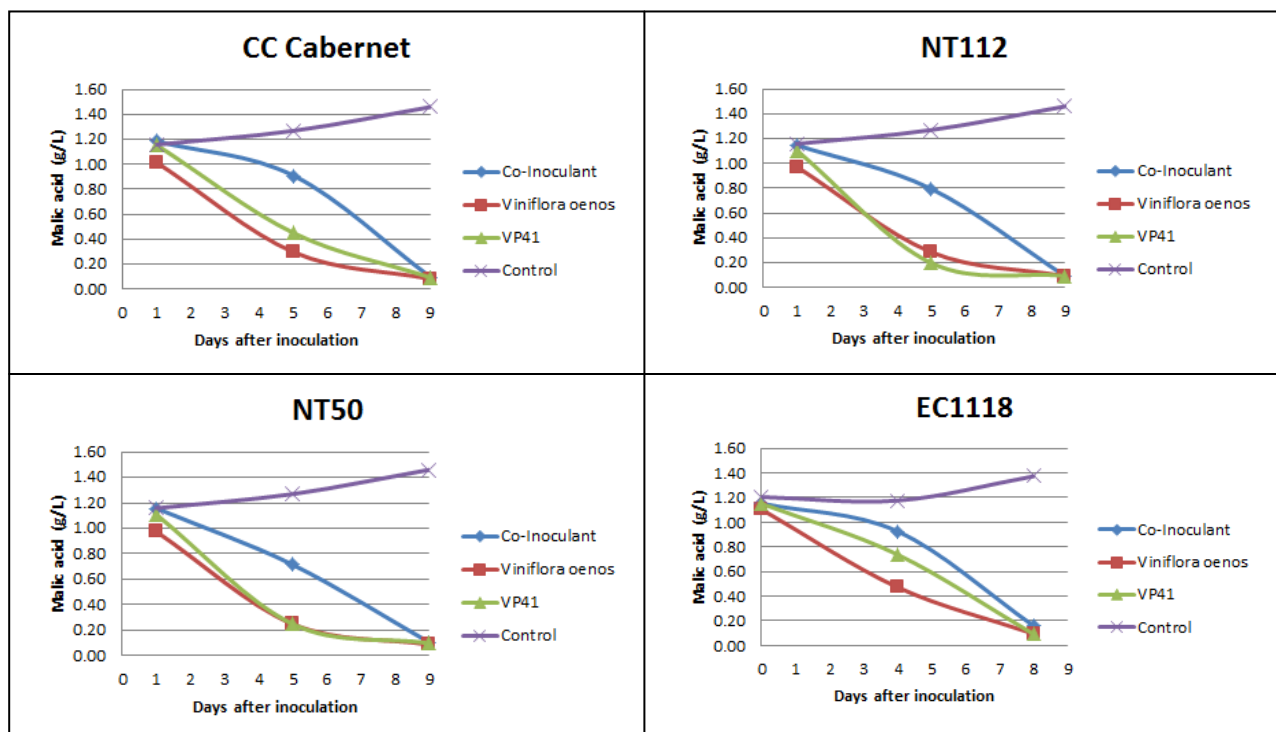


Figure 3.3 Malic acid degradation by different LAB cultures per yeast in co-inoculation observed in Merlot 2011 vintage. Data shown are averaged values of duplicate treatments (standard deviations not shown).

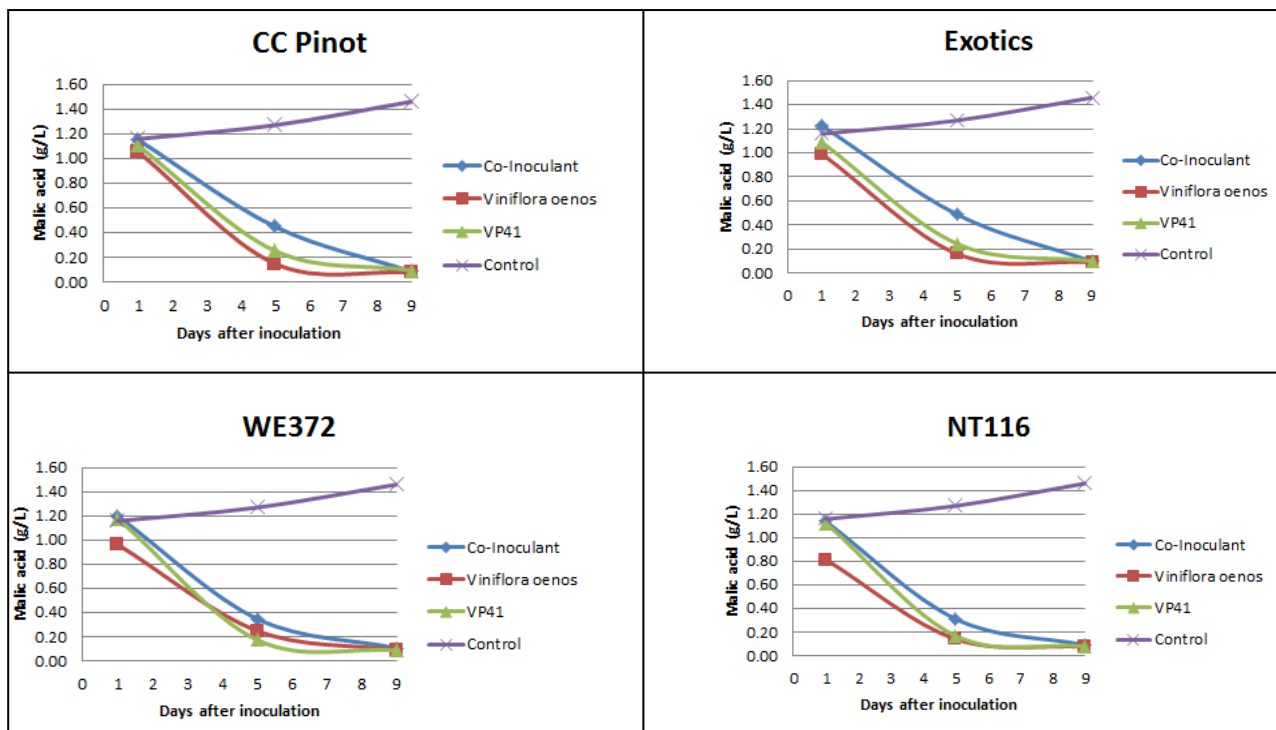


Figure 3.4 Malic acid degradation by the different LAB cultures per yeast in co-inoculation observed in Merlot 2011 vintage. Data shown are averaged values of duplicate treatments (standard deviations not shown).

The estimated time of MLF completion for the yeast strains Fermicru VR5 and Fermicru XL (that did not complete MLF in eight days) were approximately nine to ten days (**Figure 3.5**). Although the rate of malic acid degradation was faster for *Viniflora oenos* than VP41 when using Fermicru VR5, the duration of MLF was still the same (around seven days). For Fermicru XL, however, MLF completed in six days with *Viniflora oenos* and seven to eight days with VP41. This difference in malic acid degradation using the different yeast strains was not evident in the associated total LAB cell counts (**Figure 3.10**) and could therefore not explain the different categories. The classification of the different yeasts combinations could be attributed to possible yeast-bacterial interactions that inhibit or stimulate MLF.

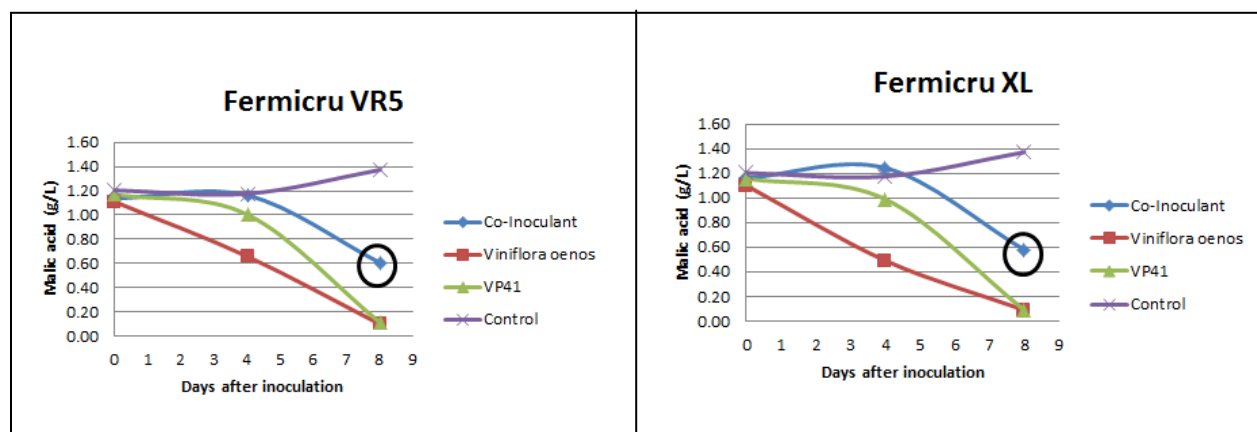


Figure 3.5 Malic acid (g/L) degradation by different LAB cultures per yeast in co-inoculation observed in Merlot 2011 vintage. Data shown are averaged values of duplicate treatments (standard deviations not shown). Black circles indicate incomplete MLF at day 8.

Classification of the yeast treatments were done according to the malic acid degradation abilities and lag phase after inoculation (not the time needed to complete MLF) of the NT 202 Co-Inoculant, used in co-inoculation. Treatments required five to eight days to complete MLF, except for Fermicru VR5 and Fermicru XL (**Table 3.8**). In general, regardless of yeast strain, *Viniflora oenos* showed faster malic acid degradation compared to NT 202 Co-Inoculant and Lalvin VP41. The fast completion of MLF in general could have been influenced by better homogenization associated with the small fermentation volumes. Faster MLF in smaller fermentation volumes were also observed in another study (Vrščaj Vodošek *et al.*, 2009).

Table 3.8 Classification of the yeast treatments co-inoculated with the NT 202 Co-Inoculant according to its malic acid degradation abilities observed in the 2011 Merlot.

Yeast treatment	Classification	Time to complete MLF (in days)
NT 202	Inhibitory towards MLF	7-8
Fermivin		7-8
CC Merlot		8
Fermirouge		7-8
Fermicru VR5		> 9
Fermicru XL		> 9
CC Cabernet	Neutral towards MLF	8
NT 112		8
NT 50		8
EC1118		7-8
CC Pinot	Stimulatory towards MLF	6-7
Exotics		6-7
WE 372		5-6
NT 116		5

Shiraz 2012

Impact of yeast strains on MLF rate

In comparison to NT 202 Co-Inoculant and the un-inoculated MLF control, Lalvin VP41 performed best in terms of malic acid degradation and lactic acid accumulation (**Figs 3.6** and **3.7**). The un-inoculated control did not undergo MLF. These results are in agreement with that found in Merlot in the 2011 vintage where, in general, Lalvin VP41 showed faster malic acid degradation compared to the NT 202 Co-Inoculant. All treatments, except for the un-inoculated MLF control, completed MLF within approximately 10 to 11 days (**Table 3.9**).

For Exotics, MLF completed in nine and seven days when co-inoculated with NT 202 Co-Inoculant and Lalvin VP41, respectively. For WE 372, MLF completed in 10 to 11 days when co-inoculated with NT 202 Co-Inoculant, and nine days when co-inoculated with Lalvin VP41. For NT 50, MLF completed in approximately five days for both NT 202 Co-Inoculant and VP41. WE 372, when co-inoculated with NT 202 Co-Inoculant, completed MLF in 10 to 11 days, and, co-inoculated with Lalvin VP41, completed MLF in six days.

No decrease in malic acid was observed for any treatments for at least one day after inoculation. This could be explained by the time needed for the inoculated LAB cultures to adapt to the grape must environment, except in the case of Exotics, where it has been shown in literature that *S. paradoxus* has the ability to degrade malic acid (Redzepovic *et al.*, 2003).

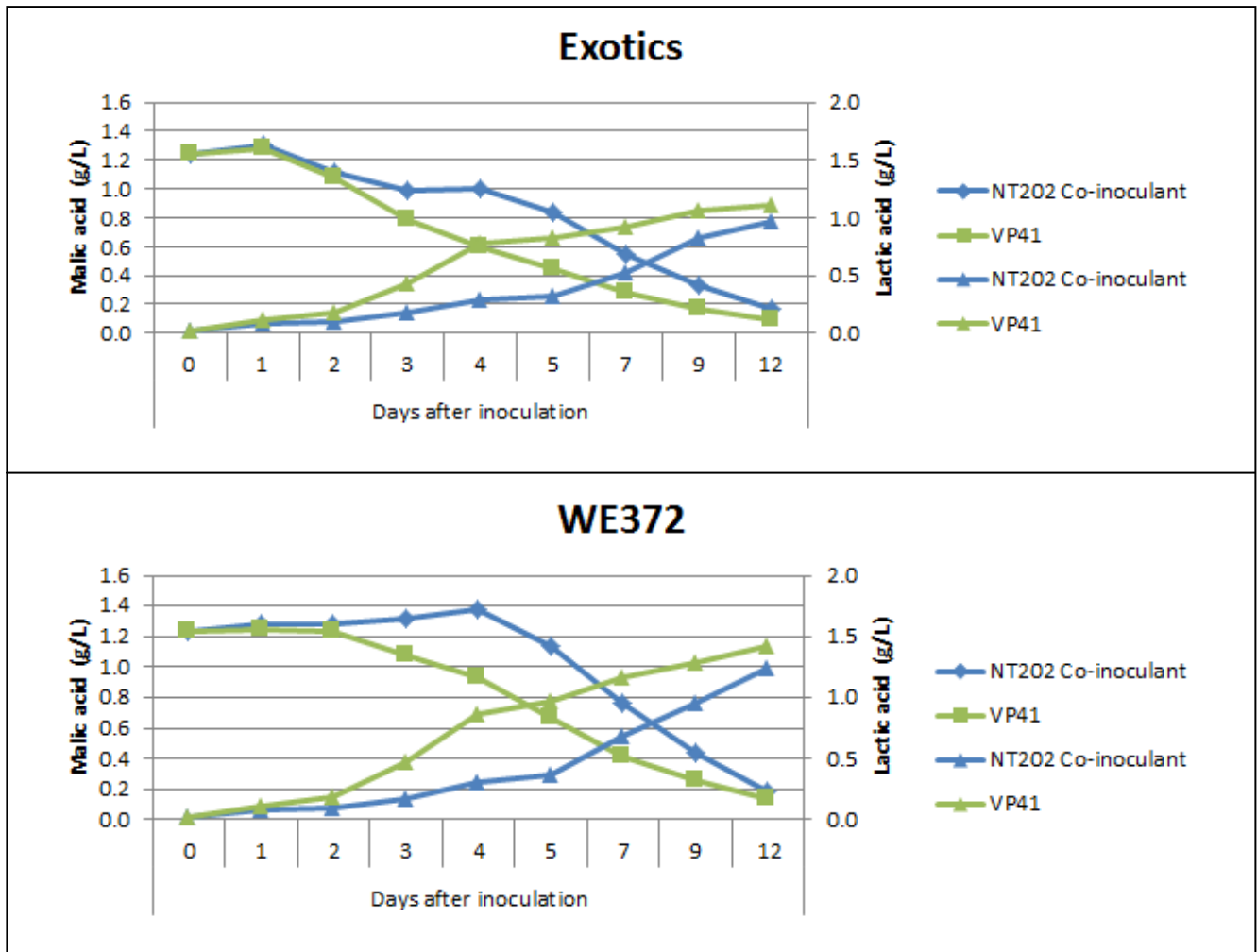


Figure 3.6 Malic acid (g/L) degradation and lactic acid accumulation (g/L) by the different LAB cultures used in co-inoculation with yeast strains Exotics and WE 372, observed in Shiraz 2012 vintage. Data shown are averaged values of triplicate treatments (standard deviations not shown). In the graphs, square data points represent the malic acid and the triangle data points represent the lactic acid values.

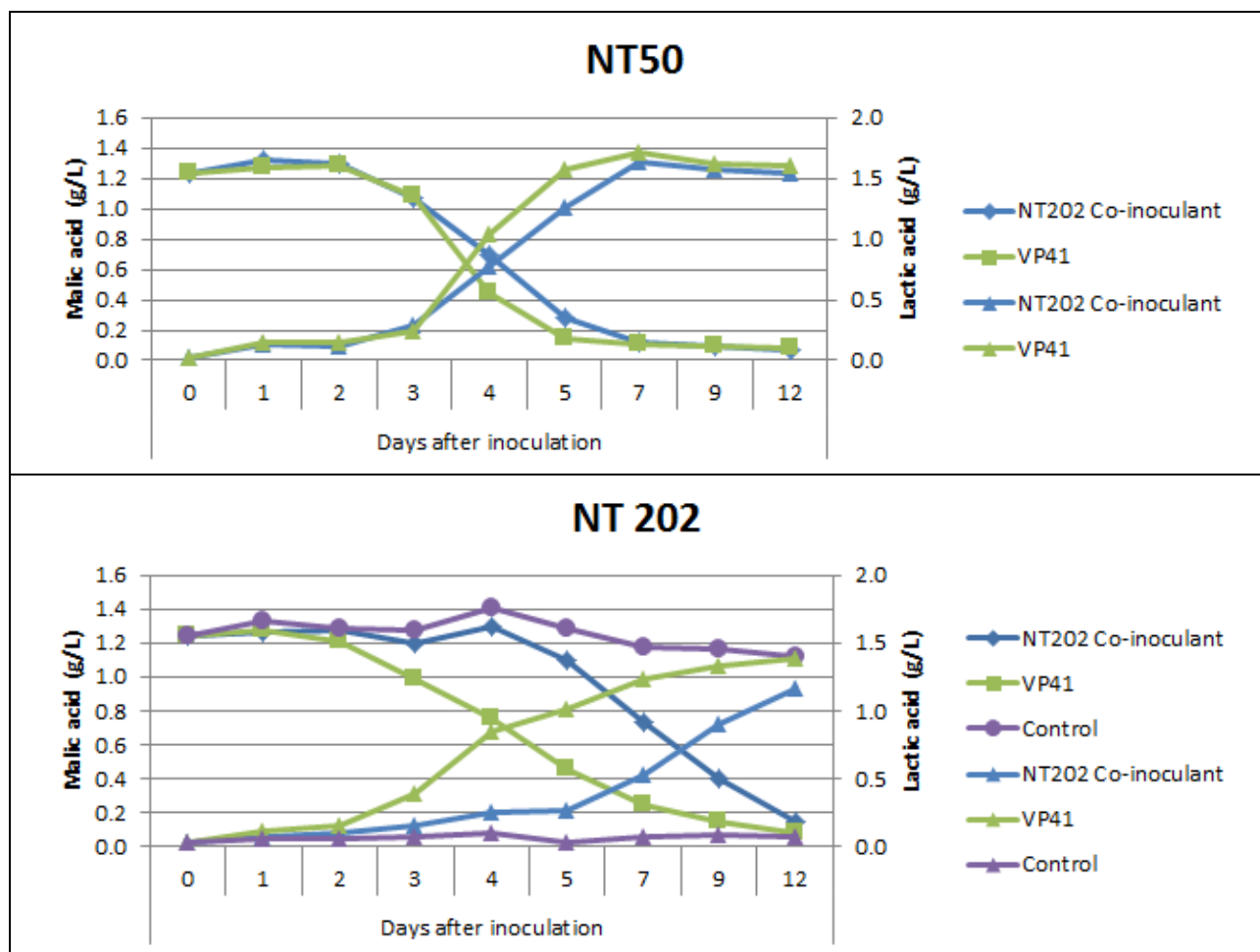


Figure 3.7 Malic acid (g/L) degradation and lactic acid (g/L) accumulation by the different LAB cultures used in co-inoculation with yeast strains NT 50 and NT 202, observed in Shiraz 2012 vintage. Data shown are averaged values of triplicate treatments (standard deviations not shown). The spontaneous MLF inoculated with NT 202 yeast was the only control conducted. In the graphs, square data points represent the malic acid and the triangle data points represent the lactic acid values.

Table 3.9 Time needed to complete MLF (in days) according to the malic acid degradation abilities of the NT 202 Co-Inoculant and Lalvin VP41, co-inoculated with four different yeast strains. The un-inoculated control did not undergo MLF and is therefore not included in the table.

Yeast treatment	MLF starter culture	
	NT 202 Co-Inoculant	Lalvin VP41
Exotics	9	7
WE 372	10-11	9
NT 50	5	4-5
NT 202	10-11	6

Impact of additives on MLF rate

The different additives that were evaluated are Extraferm, Natuferm, Claristar, OptiMalo Plus, Bactiv-Aid, Nutrivin and (DAP) as control (**Table 3.4**). Extraferm is a detoxifying agent that comprises of highly adsorbent, odourless yeast hulls that can remove toxic compounds e.g. fatty acids. Claristar is a clarifying agent that consists of mannoproteins extracted from *S. cerevisiae*. Bactiv-Aid and OptiMalo Plus are LAB nutrients that contain inactivated yeasts as well as polysaccharides and cellulose in the case of OptiMalo Plus. Natuferm and Nutrivin are yeast nutrients that include inactivated yeasts. Diammonium phosphate is a yeast nutrient that serves as the control.

Malic acid degradation for all treatments showed a rapid decrease of malic acid from day four until completion of MLF (between day 10 and 11) (**Figure 3.8**). No discernible differences in the malic acid degradation capabilities were observed between the different additives, compared to the Control (DAP), indicating that the different additives did not influence malic acid degradation. This phenomenon might be attributed to sufficient nutrient availability at onset of co-inoculated MLF.

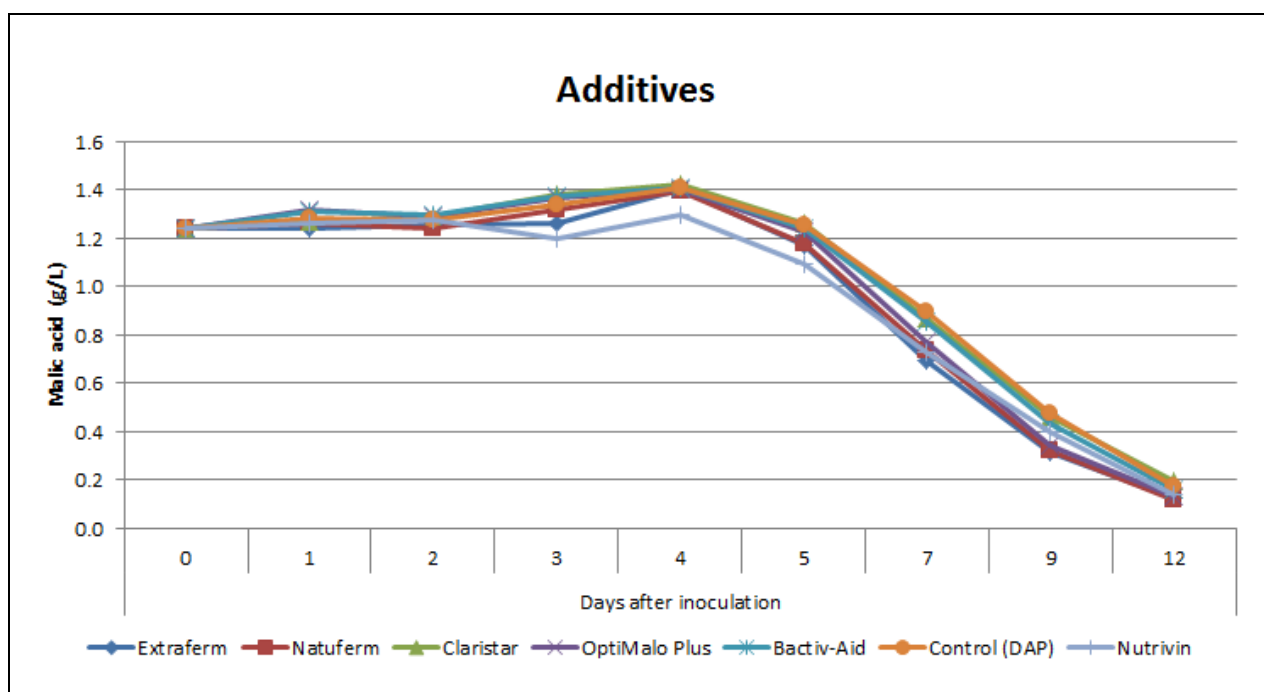


Figure 3.8 Malic acid degradation of the different treatments during fermentation in the 2012 Shiraz. Alcoholic fermentation and MLF was carried out by NT 202 yeast in co-inoculation with NT 202 Co-Inoculant. Data shown are averaged values of triplicate treatments (standard deviations not shown).

3.3.2 Microbial analysis

Chardonnay 2011

Total LAB cell counts for the different MLF starter cultures (**Figure 3.9**) showed that all treatments (except for the control) were inoculated at approximately 8.0×10^4 to 1.4×10^6 cfu/mL. Total LAB cell counts of the co-inoculated yeast treatment Exotics, regardless of the MLF starter culture, remained at approximately 10^6 cfu/mL for the entire seven weeks the fermentations were monitored. This shows the improved viability of the LAB used with Exotics. Total LAB cell counts of VIN 13, co-inoculated with NT 202 Co-Inoculant, showed a constant decrease over time until discontinuation of the fermentations. Bacterial cell numbers of the co-inoculated yeast treatment Viniflora LVCB eventually decreased to approximately 10^3 cfu/mL or below, after seven weeks, regardless of the MLF starter culture used. These low cell counts could explain why MLF for that treatment was not completed (**Figure 3.1**). Cell numbers should be between 10^6 and 10^7 cfu/mL after inoculation to ensure that L-malic acid degradation follows bacterial growth (Bauer and Dicks, 2004).

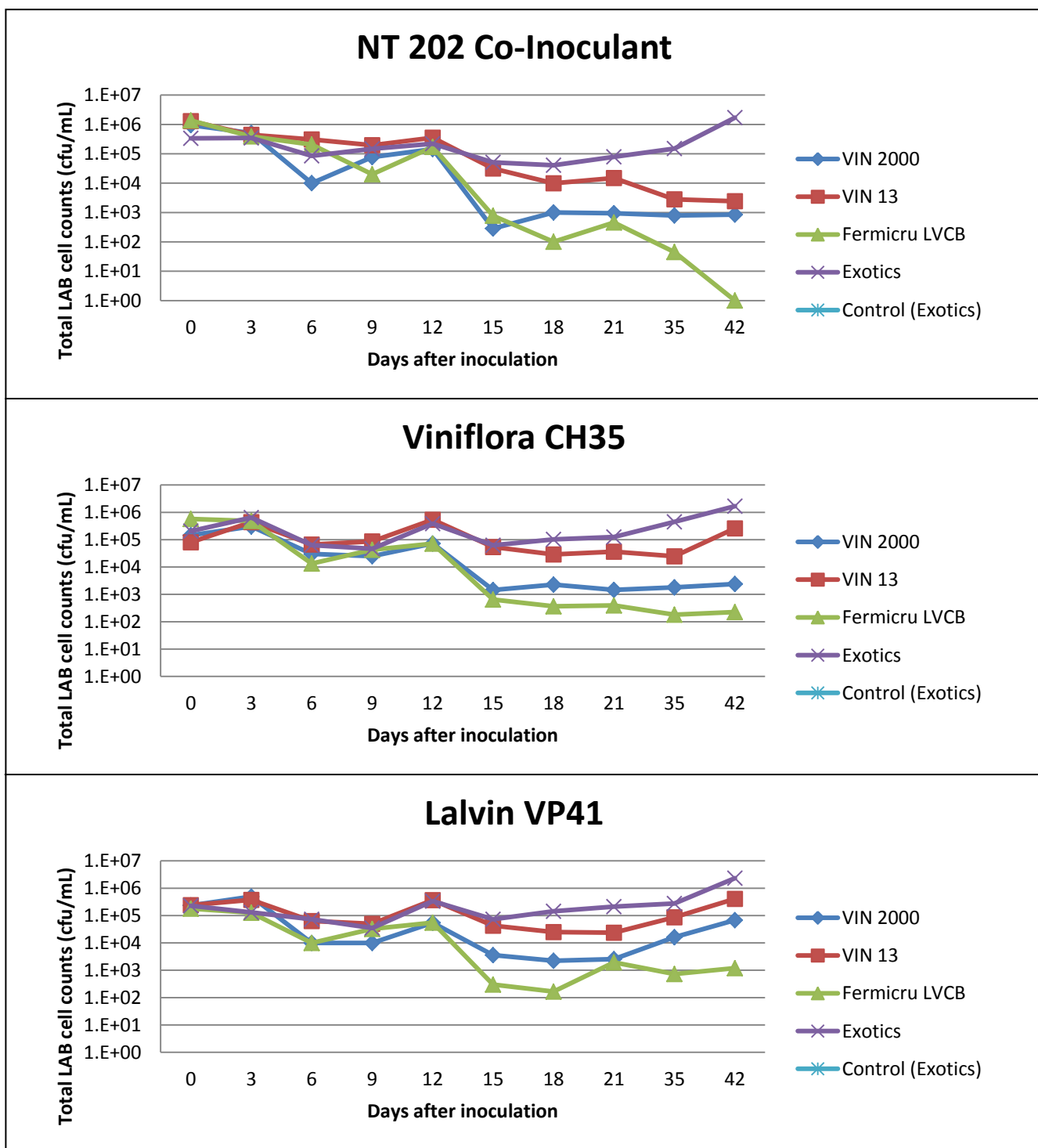


Figure 3.9 Fermentation graphs showing the bacterial growth (cfu/mL) curves of the different yeast strains co-inoculated with NT 202 Co-Inoculant, Viniflora CH35 and Lalvin VP41 in the 2012 Chardonnay. Values represent averages of triplicate treatments (standard deviations not shown).

Merlot 2011

The yeast counts show that all treatments were inoculated at approximately 10^7 to 10^8 cfu/mL, which were sufficient for successful AF (**Figure 3.10**). Yeast cell counts showed little variation between treatments throughout fermentation and thus seemed unaffected by co-inoculation with the different MLF starter cultures. This is in agreement with many other studies that found that co-inoculation had no negative effect on the yeast population or AF performance (Jussier *et al.*, 2006; Massera *et al.*, 2009; Azzolini *et al.*, 2010; Pan *et al.*, 2011; Knoll *et al.*, 2012).

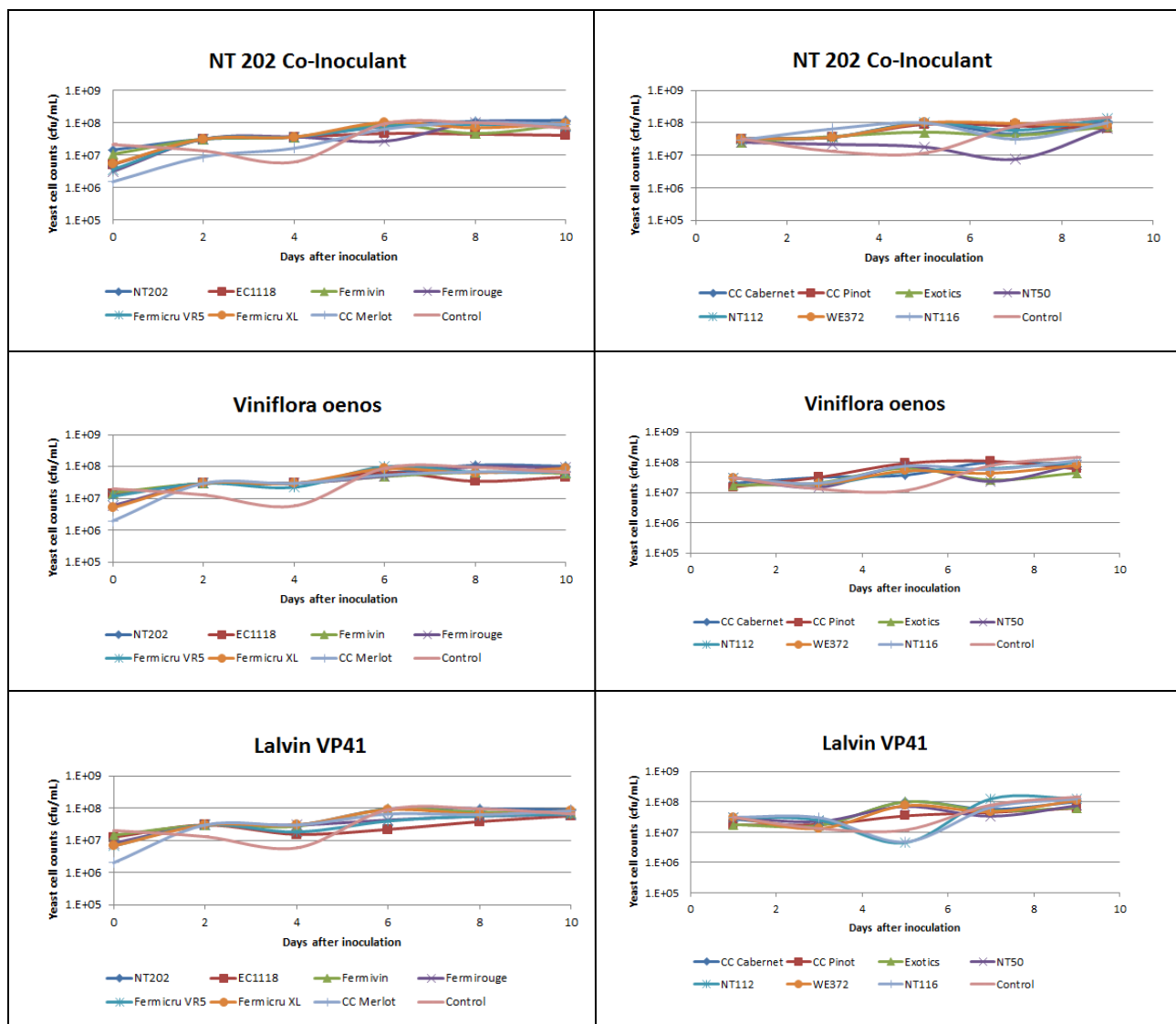


Figure 3.10 Graphs showing the yeast growth (cfu/mL) curves for NT 202 Co-Inoculant, Viniflora oenos and Lalvin VP41 in co-inoculation with the different yeast treatments, recorded at different stages of AF in Merlot in the 2011 vintage. See **Table 3.1** for treatment descriptions. Values represent averages of duplicate treatments (standard deviations not shown).

The total LAB cell counts (**Figure 3.11**) show that all treatments were inoculated at approximately 10^5 to 10^7 cfu/mL, which were sufficient to ensure successful MLF (**Figs 3.2 to 3.5**) (Bauer and Dicks, 2004). Total LAB cell counts showed little variation between treatments throughout the fermentation. Bacterial cell counts showed more variation within treatments co-inoculated with the mixed MLF starter culture NT 202 Co-Inoculant, than within treatments co-inoculated with the *O. oeni* MLF starter cultures, Viniflora oenos and Lalvin VP41. For NT 202 Co-Inoculant, LAB cell numbers ranged between approximately 10^5 and 10^7 cfu/mL, 10^6 to 10^8 cfu/mL for Viniflora oenos and 10^5 to 10^8 cfu/mL for Lalvin VP41.

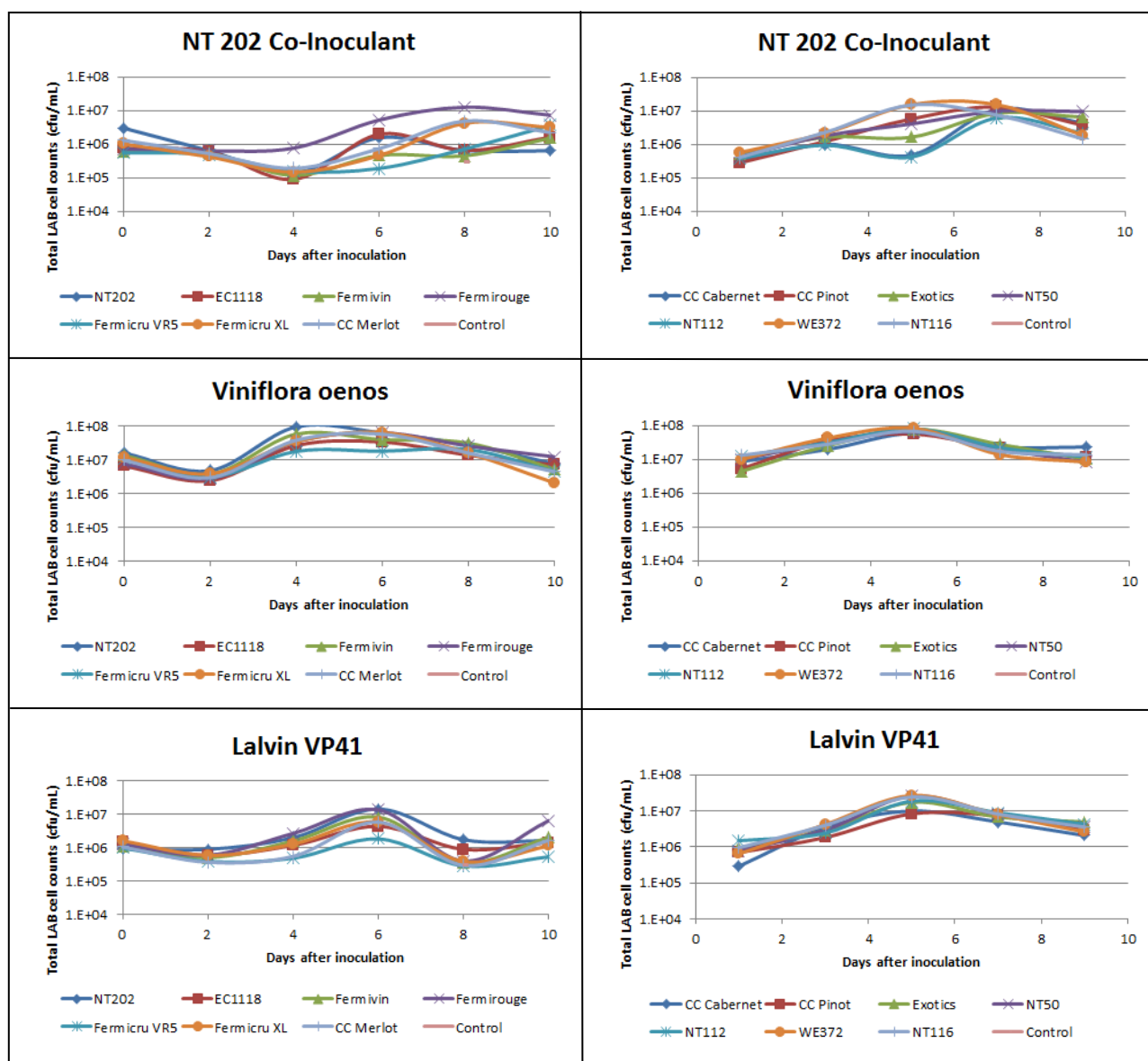


Figure 3.11 Graphs showing the bacterial growth (cfu/mL) curves for NT 202 Co-Inoculant, Viniflora oenos and Lalvin VP41 in co-inoculation with the different yeast treatments, recorded at different stages of MLF in Merlot in the 2011 vintage. See **Table 3.1** for treatment descriptions. Values represent averages of duplicate treatments (standard deviations not shown). No bacterial growth was observed for the control (not included).

Shiraz 2012**Impact of yeast-bacterial combinations on yeast and bacterial growth**

Yeast cell counts of the different yeast treatments showed little difference in cell counts between MLF starter cultures (**Figure 3.12**). The similarity of the yeast growth of the control and yeast strain NT 202 makes it evident that MLF did not affect yeast growth. This was in agreement with results found in the 2011 vintage using Merlot and several other studies previously mentioned. While the MLF control (inoculated with NT 202 yeast without bacterial inoculation), NT 202 and WE 372 showed similar fermentation kinetics, Exotics showed the same yeast growth kinetics, but with lower cell counts for both the NT 202 Co-Inoculant and Lalvin VP41. Yeast cell counts of the control, NT 202, WE 372 and Exotics peaked on day two of inoculation, whereas NT 50 cell counts peaked on day four after inoculation, regardless of the MLF starter culture used. NT 50 showed a slower increase in cell numbers initially and then a slight decrease in cell numbers after day four of co-inoculation for both NT 202 Co-Inoculant and Lalvin VP41. This difference in cell counts for the different treatments clearly shows the effect on the viability of different yeast strains during co-inoculation. These similar trends, regardless of LAB culture, were not observed in Merlot in the 2011 vintage and indicate that grape cultivar or at least differences in grape must play an important role in the growth kinetics of yeasts. All treatments showed a decrease in cell counts after the peak on day two or four after inoculation until end of AF.

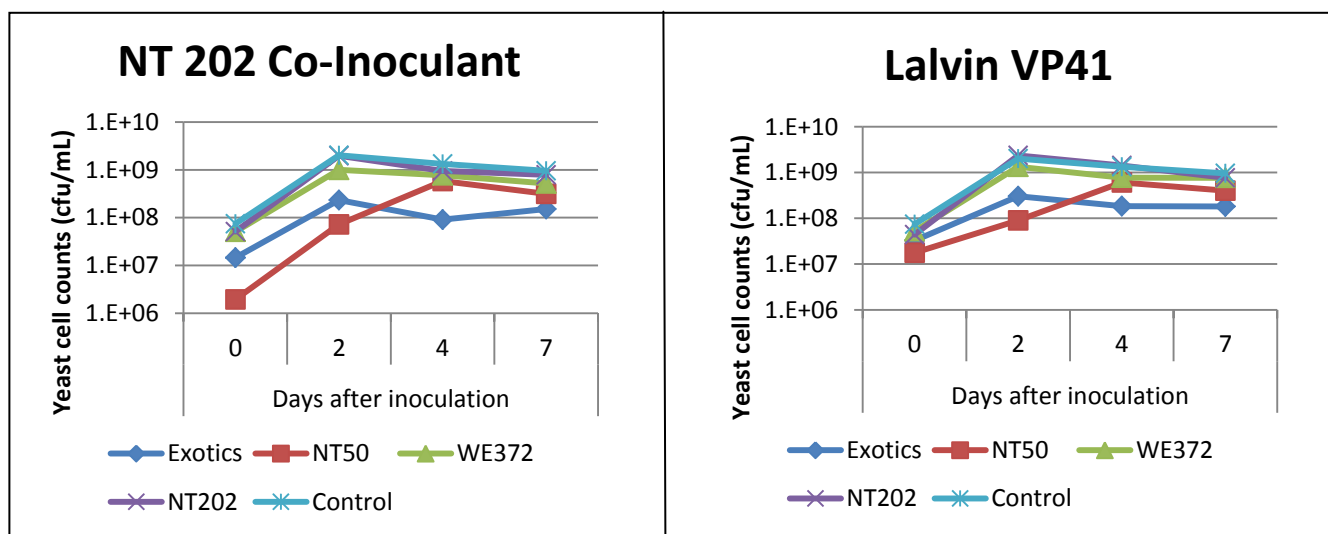


Figure 3.12 Yeast cell counts (cfu/mL) monitored for the different yeast strains co-inoculated with two MLF starter cultures, in Shiraz in 2012 for determining the impact of the yeast-bacterial combinations on the yeast growth. Values represent the average of triplicate treatments (standard deviations not shown). For the control, NT 202 yeast was inoculated with no bacterial inoculation.

Lactic acid bacteria growth kinetics of the mixed MLF starter culture, NT 202 Co-Inoculant, and the *O. oeni* starter culture, Lalvin VP41, was similar regardless of the yeast treatment (**Figure 3.13**). This shows that the yeast had little or no influence on the fermentation kinetics of the LAB during co-inoculation. Although similar LAB growth kinetics was observed for NT 202 Co-Inoculant and Lalvin VP41, regardless of yeast strain, for the major duration of MLF, NT 202 Co-Inoculant exhibited a more apparent decrease in cell counts for the first two days after inoculation. This might be explained by the presence of the *L. plantarum* in the NT 202 Co-Inoculant starter culture affecting the adaptation of the starter culture to the grape must due to possible yeast-bacterial interactions. The *L. plantarum* might be stimulated more or inhibited less than the *O. oeni* by some metabolites either produced by the yeasts or present in the grape must. Thereafter variations in LAB cell counts regardless of yeast strains were less apparent. This indicated that the possible yeast-bacterial interaction at the onset of MLF due to the presence of *L. plantarum* was overcome to such an extent that the presence of *L. plantarum* did not affect LAB cell counts for the remainder of MLF. The most variation in LAB cell counts co-inoculated with the different yeast strains occurred during mid MLF. The drop in cell counts four days after inoculation might be due to experimental error. No LAB growth was observed for the un-inoculated MLF control. Yeast treatment Exotics showed the least fluctuations in LAB cell counts, regardless of the MLF starter culture used. NT 50 showed a decrease in LAB cell numbers at the later stages of MLF (**Table 3.9**). WE 372 and NT 202 showed similar LAB growth kinetics, regardless of the MLF starter culture used.

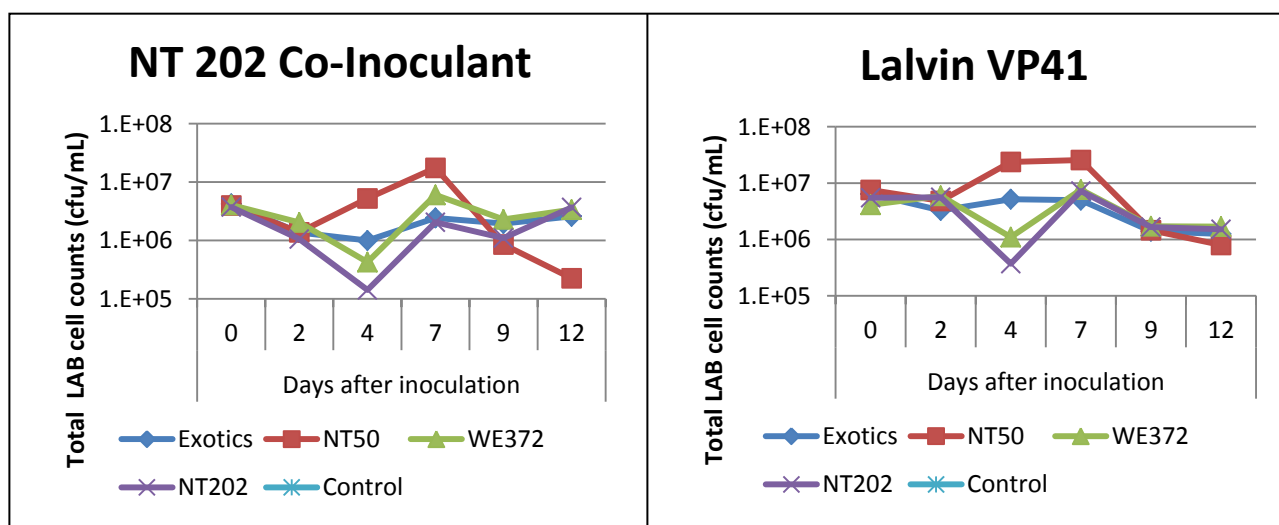


Figure 3.13 Bacterial cell counts of the two MLF starter cultures co-inoculated with the different yeast strains in Shiraz in 2012. Values represent averages of triplicate treatments. For the control NT 202 yeast was inoculated with no bacterial inoculation. No bacterial growth was observed for the control (not included).

Impact of additives on yeast and bacterial growth

Little variation in LAB cell counts were observed between the different treatments (**Figure 3.14**). All treatments showed a slight decrease in LAB cell numbers over the 12 days monitored. This trend was also evident in the malic acid degradation shown in **Figure 3.8**.

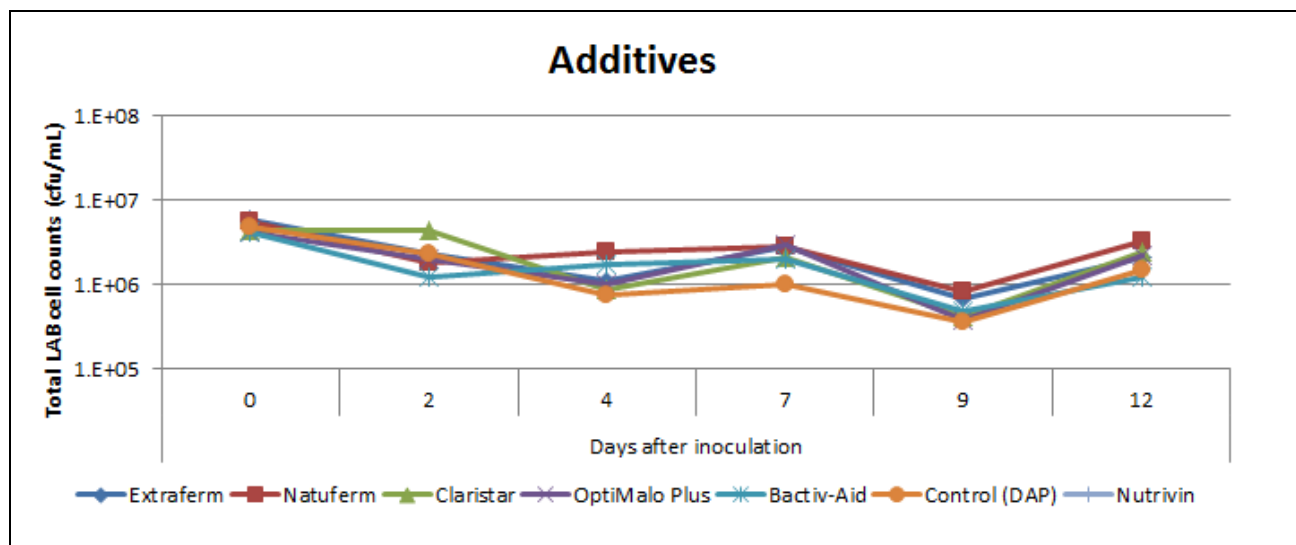


Figure 3.14 Bacterial cell counts of the NT 202 Co-Inoculant that was co-inoculated with yeast NT 202 in Shiraz 2012 vintage. Values represent averages of triplicate treatments (standard deviations not shown).

3.3.3 Volatile aroma compounds

In the present study it was found that not all volatile compounds were produced in all the cultivars above detection limits. This was also observed in a study done by Lerm (2010). In general, MLF treatments in the Shiraz 2012 produced lower concentrations of total esters for yeast treatment NT 50 and WE 372 whereas similar concentrations were produced for yeast treatments NT 202 and Exotics, compared to the 2011 Merlot. Overall, the Merlot 2011 produced lower concentrations of total volatile fatty acids as well as total higher alcohols. Even though MLF treatment, cultivar and vintage influence the production of these volatile compounds, compounds that show noticeable trends between the different MLF treatments and yeast treatments for a MLF treatment will be the focus and will be discussed individually with regard to cultivar (**Tables 3.10 to 3.12**). Data generated using GC-FID and GC-MS were not subjected to univariate statistical analysis and results discussed only refer to trends observed in the data. Associated aroma contributions of some compounds are indicated in brackets.

Merlot 2011

The GC-generated results of the 2011 Merlot were used to determine the changes in volatile composition that could be attributed to the different yeast/MLF starter culture combinations and to study trends in the volatile profile of the wines.

Esters

Malolactic fermentation resulted in variations between yeast treatments as well as MLF treatments compared to the un-inoculated MLF control for most of the esters produced. Ethyl propionate and ethyl-2-methylpropanoate, both of which impart a fruity character to wine (Escudero *et al.*, 2007), were not detected for any treatment co-inoculated with *Viniflora oenos* or Lalvin VP41 (**Tables 3.10** and **3.12**). The latter two compounds were, however, detected for some yeast treatments in combination with the NT 202 Co-Inoculant, but in low quantities. 2-Methyl-propyl acetate and ethyl caprate (ethyl decanoate) were only detected for some treatments. Ethyl acetate was quantitatively the predominant ester after completion of MLF. This was also found by Lerm (2010). It is often an important contributor to wine aroma by giving desirably fruity characters, in low concentrations, and solvent or nail varnish-like aromas, at high concentrations (Sumbly *et al.*, 2010). NT 112 and WE 372 showed the highest concentrations of ethyl acetate whereas EC1118, Fermicru XL and NT 50 showed the lowest concentrations, regardless of the MLF starter culture used. Although higher concentrations of ethyl butyrate (fruity) (Escudero *et al.*, 2007) were observed for Fermicru XL, Fermirouge, Fermicru VR5 and WE 372, irrespective of the MLF starter culture used, co-inoculation with NT 202 Co-Inoculant resulted in a trend of higher concentrations, compared to the remaining MLF starter cultures (**Figure 3.15**). The highest concentrations of isoamyl acetate (banana, fruity) (Sumbly *et al.*, 2010) were observed for Fermirouge, Fermicru VR5 and CC Merlot whereas the lowest concentrations were observed for EC1118, regardless of the MLF starter culture used. For NT 202, MLF showed decreased concentrations of isoamyl acetate. Studies done by Herjavec *et al.* (2001) as well as Jeromel *et al.* (2008) found decreased levels of isoamyl acetate after completion of MLF. Changes in ethyl hexanoate were strain dependent as previously found by Malherbe (2010) in a study done on Pinotage and Shiraz using sequential inoculation. Malolactic fermentation led to decreased concentrations of ethyl hexanoate (fruity, strawberry, green apple, anise) (Sumbly *et al.*, 2010) for yeast NT 202. The highest concentrations of ethyl hexanoate were observed for Fermicru VR5 and Fermicru XL, when co-inoculated with NT 202 Co-Inoculant. Malolactic fermentation clearly led to increased ethyl lactate concentrations in the final wine, compared to the un-inoculated MLF control for all yeast treatments, of which WE 372 showed the highest concentration (**Figure 3.15**). Increased ethyl lactate concentrations due to MLF are in accordance with previous studies done by Lerm (2010)

and Malherbe (2010). Ethyl lactate is formed via the esterification of lactic acid and ethanol and production thereof depend on the amount of lactic acid formed during MLF. Ethyl lactate is favourable to wine aroma due to its fruity, buttery and creamy aromas and contribution to the mouthfeel of the wine (Lerm *et al.*, 2010). No discernible trends were observed for ethyl caprylate. For ethyl caprylate (ethyl octanoate) (sweet, fruity, ripe fruit, burned, beer) (Sumbly *et al.*, 2010), the highest concentrations, when co-inoculated with NT 202 Co-Inoculant, were observed for Fermirouge, Fermicru XL and CC Merlot. Co-inoculation with *Viniflora oenos* showed that Exotics and WE 372 lead to higher ethyl caprylate concentrations whereas co-inoculation with *Lalvin VP41* showed that Fermicru XL and Exotics lead to the highest concentrations. Diethyl succinate is another important ester relating to MLF that was observed to be dependent on yeast strain rather than LAB strain. Yeast treatments CC Pinot, CC Cabernet and Fermirouge showed the highest concentrations of diethyl succinate (fruity, fermented, floral) (Sumbly *et al.*, 2010) that were all higher than concentrations observed for the un-inoculated MLF control. For NT 202 MLF clearly led to increased levels of diethyl succinate. This supported previous findings by Lerm (2010) and Malherbe (2010). For 2-phenylethyl acetate (roses) (Sumbly *et al.*, 2010), the highest concentrations were observed for CC Cabernet, when co-inoculated with NT 202 Co-Inoculant, and for CC Pinot and Exotics, when co-inoculated with *Viniflora oenos* and *Lalvin VP41*.

Table 3.10 Esters, higher alcohols and volatile fatty acids (mg/L) measured in 2011 in Merlot after completion of co-inoculated MLF using the Anchor NT 202 Co-Inoculant. Concentrations represent the average of duplicate treatments (standard deviations not shown), each analysed in duplicate by GC-FID (nd: not detected).

Esters	NT 202 Co-Inoculant														Control
	NT 202	EC 1118	Fermivin	Fermirouge	Fermicru VR5	Fermicru XL	CC Merlot	CC Cabernet	CC Pinot	Exotics	NT 50	NT 112	WE 372	NT 116	NT 202
Ethyl Acetate	63.58	58.44	71.42	82.73	70.71	55.19	78.78	64.46	76.22	76.54	47.74	101.57	101.64	71.29	75.76
Ethyl Propionate	2.10	1.91	nd	nd	3.12	nd	nd	2.13	2.24	2.02	nd	nd	nd	nd	nd
Ethyl-2-methylpropanoate	nd	0.53	1.09	1.53	1.95	0.72	0.62	0.83	1.11	0.85	nd	nd	nd	nd	nd
2-Methyl-propyl acetate	nd	nd	0.22	0.22	0.23	0.22	0.22	nd	0.22	nd	nd	0.21	nd	nd	nd
Ethyl Butyrate	0.19	0.24	0.18	0.30	0.26	0.32	0.24	0.25	0.14	0.20	0.16	0.17	0.24	0.16	0.21
Isoamyl Acetate	0.13	0.13	0.15	0.20	0.21	0.18	0.17	0.20	0.14	0.13	0.12	0.14	0.15	0.13	0.15
Ethyl Hexanoate	0.39	0.42	0.48	0.51	0.72	0.78	0.57	0.43	0.37	0.46	0.43	0.44	0.50	0.40	0.51
Ethyl Lactate	64.68	37.75	61.28	69.23	72.04	64.53	73.63	76.61	41.40	57.23	104.49	106.61	182.42	91.19	5.48
Ethyl Caprylate	0.15	0.14	0.16	0.21	0.17	0.19	0.20	0.19	0.13	0.15	0.12	0.12	0.13	0.11	0.15
Ethyl caprate	nd	nd	nd	0.02	0.07	0.19	nd	0.03	0.25	nd	0.01	0.02	nd	0.00	nd
Diethyl succinate	3.30	4.13	3.85	5.61	5.23	6.05	3.04	5.79	6.49	5.13	2.81	2.73	2.92	2.62	3.20
2-Phenylethyl Acetate	0.49	0.49	0.51	0.54	0.53	0.53	0.49	0.56	0.52	0.53	0.48	0.49	0.50	0.48	0.50
Total Esters	135.02	104.18	139.33	161.10	155.23	128.89	157.97	151.48	129.22	143.24	156.37	212.51	288.51	166.40	85.96
Volatile Fatty Acids															
Acetic Acid	71.28	76.43	102.88	191.35	154.97	137.25	105.75	151.15	89.11	57.53	85.59	136.20	153.40	128.40	66.48
Propionic Acid	1.91	2.24	1.97	2.04	2.37	2.10	1.82	2.35	2.59	2.09	2.12	3.20	3.19	2.65	2.08
Isobutyric Acid	1.77	2.46	3.51	7.32	4.43	4.17	2.27	3.32	4.69	3.20	2.32	2.20	2.86	1.93	1.96
Butyric acid	0.16	0.19	0.19	0.21	0.22	0.23	0.20	0.19	0.15	0.19	0.18	0.18	0.21	0.16	0.16
Isovaleric acid	0.59	0.34	0.72	1.16	0.58	0.77	0.98	0.64	0.33	0.77	0.45	0.39	0.78	0.52	0.63
Valeric Acid	0.05	0.06	0.03	0.11	0.05	0.04	0.01	0.17	0.12	0.10	nd	0.16	0.18	0.07	0.06
Hexanoic Acid	1.96	2.08	2.53	2.63	2.76	3.17	2.75	2.36	1.82	2.31	1.59	1.65	2.07	1.36	1.88
Decanoic Acid	0.05	nd	nd	0.11	0.10	0.25	0.65	nd	nd	nd	nd	nd	nd	nd	0.01
Total Fatty Acids	77.77	83.80	111.83	204.91	165.47	147.97	114.44	160.17	98.81	66.19	92.25	143.98	162.68	135.08	73.24
Higher alcohols															
Methanol	114.16	98.11	108.95	42.03	142.60	75.59	64.99	134.15	129.96	114.66	56.13	99.14	69.98	78.57	112.31
Propanol	62.01	54.02	28.54	33.17	31.95	35.88	29.17	35.77	54.68	49.52	30.98	112.33	83.60	67.83	68.11
Isobutanol	40.72	53.92	65.92	97.18	95.27	57.86	62.53	41.28	118.21	58.27	55.50	51.09	50.43	48.97	46.88
Butanol	1.65	1.62	0.78	0.65	1.31	1.19	1.10	4.64	1.44	4.71	2.54	1.63	1.69	2.35	1.78
Isoamyl Alcohol	361.63	415.68	426.59	571.93	590.48	646.14	523.96	617.61	593.64	514.31	358.55	350.63	427.69	347.56	354.82
4-Methyl-1-pentanol	0.27	0.29	0.31	0.32	0.29	0.31	0.34	0.35	0.28	0.33	0.27	0.25	0.29	0.25	0.26
3-Methyl-1-pentanol	0.38	0.37	0.40	0.48	0.50	0.46	0.46	0.53	0.44	0.65	0.36	0.34	0.44	0.34	0.40
Hexanol	2.41	2.42	2.12	2.38	2.93	2.70	2.45	2.28	2.06	2.29	0.09	1.78	1.91	1.98	2.66
3-ethoxy-1-propanol	3.60	5.44	1.55	1.95	1.88	2.02	3.12	3.97	6.11	3.01	2.45	16.40	6.70	4.92	3.50
2-Phenylethanol	67.16	75.43	76.10	81.81	107.45	115.24	69.32	130.03	150.17	130.56	86.21	53.59	74.83	57.38	65.29
Total Higher Alcohols	654.00	707.29	711.26	831.91	974.67	937.38	757.44	970.62	1056.97	878.31	593.07	687.17	717.56	610.16	656.03

Table 3.11 Esters, higher alcohols and volatile fatty acids (mg/L) measured in 2011 in Merlot after completion of co-inoculated MLF using *Viniflora oenos*. Concentrations represent the average of duplicate treatments (standard deviations not shown), each analysed in duplicate by GC-FID (nd: not detected).

Esters	Viniflora oenos														Control
	NT 202	E-1118	Fermivin	Fermirouge	Fermicru VR5	Fermicru XL	CC Merlot	CC Cabernet	CC Pinot	Exotics	NT 50	NT 112	WE 372	NT 116	NT 202
Ethyl Acetate	63.34	59.19	74.54	83.59	90.46	62.86	97.88	80.51	89.50	80.48	67.71	116.38	103.62	93.46	75.76
Ethyl Propionate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ethyl-2-methylpropanoate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-Methyl-propyl acetate	nd	nd	0.21	0.22	0.21	0.21	0.21	0.21	0.22	0.21	nd	0.21	nd	nd	nd
Ethyl Butyrate	0.12	0.15	0.17	0.21	0.21	0.19	0.20	0.17	0.15	0.21	0.21	0.21	0.25	0.14	0.21
Isoamyl Acetate	0.12	0.12	0.14	0.21	0.18	0.15	0.15	0.18	0.17	0.16	0.14	0.16	0.18	0.13	0.15
Ethyl Hexanoate	0.40	0.40	0.46	0.50	0.48	0.45	0.47	0.43	0.41	0.51	0.55	0.50	0.61	0.38	0.51
Ethyl Lactate	114.34	42.63	80.86	84.03	57.73	53.63	92.88	86.60	79.77	103.10	73.34	73.69	157.74	40.43	5.48
Ethyl Caprylate	0.12	0.13	0.15	0.17	0.15	0.15	0.17	0.14	0.13	0.20	0.15	0.16	0.22	0.13	0.15
Ethyl caprate	nd	nd	nd	nd	nd	nd	nd	0.00	nd	0.14	0.10	nd	0.05	nd	nd
Diethyl succinate	3.19	3.75	4.24	4.95	3.75	3.79	2.88	5.63	6.39	3.96	4.89	3.39	2.19	2.97	3.20
2-Phenylethyl Acetate	0.47	0.48	0.50	0.52	0.52	0.49	0.50	0.53	0.54	0.53	0.51	0.50	0.51	0.49	0.50
Total Esters	182.09	106.86	161.29	174.38	153.69	121.92	195.35	174.39	177.27	189.50	147.59	195.19	265.37	138.13	85.96
Volatile Fatty Acids															
Acetic Acid	141.72	126.25	197.14	218.24	214.88	146.99	173.09	185.33	129.43	95.00	151.59	196.30	180.18	151.90	66.48
Propionic Acid	2.54	2.28	1.99	1.71	1.74	1.52	1.78	2.19	2.14	1.51	2.52	3.22	2.22	2.54	2.08
Isobutyric Acid	2.06	2.61	4.27	6.15	3.22	2.55	2.21	2.87	4.06	2.24	3.63	2.67	2.06	1.97	1.96
Butyric acid	0.16	0.17	0.18	0.18	0.17	0.16	0.17	0.16	0.16	0.13	0.21	0.18	0.22	0.15	0.16
Isovaleric acid	0.15	0.03	0.48	0.69	0.40	0.51	0.69	0.50	0.20	0.83	0.64	0.39	0.78	0.41	0.63
Valeric Acid	0.07	0.02	0.02	nd	0.01	nd	nd	0.11	0.10	0.09	0.00	0.08	0.08	0.02	0.06
Hexanoic Acid	1.39	1.80	2.26	2.42	2.28	2.10	2.38	2.00	1.91	1.66	2.61	1.94	1.46	1.50	1.88
Decanoic Acid	0.25	nd	nd	nd	nd	nd	nd	nd	nd	0.02	0.07	0.02	0.04	nd	0.01
Total Fatty Acids	148.34	133.16	206.33	229.39	222.70	153.83	180.31	193.18	137.99	101.48	161.27	204.80	187.04	158.51	73.24
Higher Alcohols															
Methanol	195.01	92.75	97.79	74.21	130.86	92.45	75.24	111.04	96.08	118.50	107.36	118.80	110.74	98.73	112.31
Propanol	79.76	53.94	37.43	27.90	29.74	26.51	30.88	55.70	51.35	52.92	21.65	105.08	61.83	66.87	68.11
Isobutanol	42.46	52.61	75.16	110.65	79.36	59.37	72.35	59.56	103.12	66.68	65.23	67.49	51.90	46.60	46.88
Butanol	2.00	1.55	0.76	0.86	0.86	0.95	0.79	3.74	1.64	4.71	2.60	1.77	1.68	1.80	1.78
Isoamyl Alcohol	334.39	354.60	407.54	511.33	446.32	493.17	440.29	506.04	370.18	567.45	467.12	383.21	450.68	322.93	354.82
4-Methyl-1-pentanol	0.25	0.26	0.27	0.32	0.28	0.28	0.29	0.30	0.30	0.34	0.30	0.26	0.30	0.25	0.26
3-Methyl-1-pentanol	0.32	0.32	0.35	0.45	0.39	0.37	0.36	0.51	0.51	0.67	0.41	0.33	0.39	0.33	0.40
Hexanol	1.80	1.95	2.06	2.35	1.12	0.10	2.62	2.24	2.34	2.42	2.82	1.06	2.32	0.09	2.66
3-ethoxy-1-propanol	4.87	4.61	1.61	1.59	1.47	1.55	1.83	2.98	5.60	2.82	2.30	9.05	3.98	3.70	3.50
2-Phenylethanol	61.80	64.21	77.03	76.27	86.93	80.79	69.22	111.67	155.91	129.88	119.59	58.74	73.27	57.51	65.29
Total Higher Alcohols	722.65	626.80	700.01	805.90	777.33	755.53	693.87	853.77	787.04	946.38	789.38	745.78	757.09	598.81	656.03

Table 3.12 Esters, higher alcohols and volatile fatty acids (mg/L) measured in 2011 in Merlot after completion of co-inoculated MLF using Lalvin VP41. Concentrations represent the average of duplicate treatments (standard deviations not shown), each analysed in duplicate by GC-FID (nd: not detected).

Esters	Lalvin VP41														Control
	NT 202	EC 1118	Fermivin	Fermirouge	Fermicru VR5	Fermicru XL	CC Merlot	CC Cabernet	CC Pinot	Exotics	NT 50	NT 112	WE 372	NT 116	NT 202
Ethyl Acetate	65.15	64.74	77.76	86.30	93.29	68.72	89.13	78.13	68.61	81.78	62.65	103.65	90.52	89.55	75.76
Ethyl Propionate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ethyl-2-methylpropanoate	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-Methyl-propyl acetate	nd	0.21	0.21	0.22	0.21	0.21	0.21	0.21	0.22	nd	nd	0.11	0.11	0.11	nd
Ethyl Butyrate	0.19	0.20	0.22	0.21	0.21	0.23	0.19	0.18	0.21	0.22	0.17	0.19	0.24	0.18	0.21
Isoamyl Acetate	0.14	0.12	0.17	0.23	0.20	0.16	0.17	0.19	0.17	0.16	0.12	0.15	0.17	0.14	0.15
Ethyl Hexanoate	0.45	0.38	0.53	0.59	0.52	0.55	0.50	0.53	0.51	0.65	0.47	0.43	0.54	0.51	0.51
Ethyl Lactate	70.36	31.03	83.59	60.54	77.45	58.83	58.42	74.16	56.07	71.66	43.55	46.44	121.69	62.12	5.48
Ethyl Caprylate	0.15	0.13	0.16	0.18	0.18	0.21	0.17	0.16	0.15	0.19	0.16	0.14	0.17	0.14	0.15
Ethyl caprate	nd	nd	nd	nd	nd	0.10	nd	nd	0.01	0.02	0.06	nd	nd	0.01	nd
Diethyl succinate	3.82	3.12	5.18	6.07	4.56	4.00	3.84	6.03	6.30	6.41	3.65	3.64	3.06	3.63	3.20
2-Phenylethyl Acetate	0.49	0.48	0.51	0.54	0.54	0.50	0.50	0.54	0.54	0.54	0.49	0.50	0.50	0.49	0.50
Total Esters	140.75	100.41	168.34	154.89	177.16	133.51	153.13	160.14	132.80	161.65	111.33	155.24	217.00	156.89	85.96
Volatile Fatty Acids															
Acetic Acid	100.58	110.27	201.55	177.88	220.03	122.96	158.04	148.86	100.18	104.79	120.76	143.70	163.96	133.58	66.48
Propionic Acid	2.19	2.38	2.07	1.95	2.38	1.77	2.58	2.37	2.16	2.65	2.19	2.95	3.17	2.90	2.08
Isobutyric Acid	2.07	2.54	4.99	7.43	4.32	2.18	3.15	3.30	3.58	4.38	3.05	2.69	3.16	2.55	1.96
Butyric acid	0.17	0.17	0.21	0.20	0.20	0.19	0.20	0.17	0.16	0.21	0.17	0.17	0.19	0.17	0.16
Isovaleric acid	0.55	0.07	0.63	0.79	0.56	0.95	0.81	0.50	0.11	0.54	0.43	0.63	0.37	0.35	0.63
Valeric Acid	0.05	0.03	0.03	0.01	0.04	0.02	0.03	0.14	0.08	0.13	0.00	0.06	0.08	0.05	0.06
Hexanoic Acid	2.02	1.42	2.81	2.85	2.49	1.64	2.56	2.19	1.88	2.60	1.88	2.05	1.87	1.74	1.88
Decanoic Acid	nd	1.13	nd	nd	nd	nd	nd	0.07	0.05	0.09	0.01	0.04	0.03	0.02	0.01
Total Fatty Acids	107.62	118.00	212.28	191.12	230.02	129.71	167.37	157.59	108.20	115.39	128.49	152.28	172.82	141.36	73.24
Higher Alcohols															
Methanol	135.80	172.88	137.80	75.28	150.33	62.71	92.98	134.08	100.42	119.78	103.56	117.26	94.07	100.49	112.31
Propanol	73.26	85.48	37.00	34.54	40.60	34.35	49.06	46.95	36.92	58.75	27.60	87.89	65.02	59.86	68.11
Isobutanol	49.75	62.28	85.21	124.49	77.12	68.95	75.35	61.75	89.22	71.68	67.80	63.85	55.26	53.68	46.88
Butanol	2.31	1.80	1.02	1.27	1.20	1.23	1.16	4.18	1.52	5.10	2.57	1.70	1.69	2.16	1.78
Isoamyl Alcohol	411.15	381.23	513.73	180.65	501.85	509.88	493.89	548.18	626.73	577.30	399.64	403.48	419.07	375.89	354.82
4-Methyl-1-pentanol	0.28	0.29	0.31	0.34	0.32	0.30	0.30	0.32	0.31	0.34	0.28	0.26	0.28	0.26	0.26
3-Methyl-1-pentanol	0.42	0.29	0.30	0.53	0.32	0.41	0.40	0.56	0.57	0.66	0.36	0.36	0.42	0.37	0.40
Hexanol	2.78	2.05	0.12	0.11	0.12	2.41	0.12	2.45	2.57	2.67	2.26	0.10	2.05	2.26	2.66
3-ethoxy-1-propanol	3.72	4.52	2.06	1.78	1.81	1.77	2.44	3.26	4.19	3.64	1.92	6.76	4.37	3.61	3.50
2-Phenylethanol	76.31	47.23	97.49	93.57	101.46	102.20	88.03	128.33	150.93	145.92	94.71	66.75	71.39	72.53	65.29
Total Higher Alcohols	755.79	758.06	875.04	512.56	875.12	784.21	803.75	930.05	1013.39	985.84	700.71	748.41	713.64	671.10	656.03

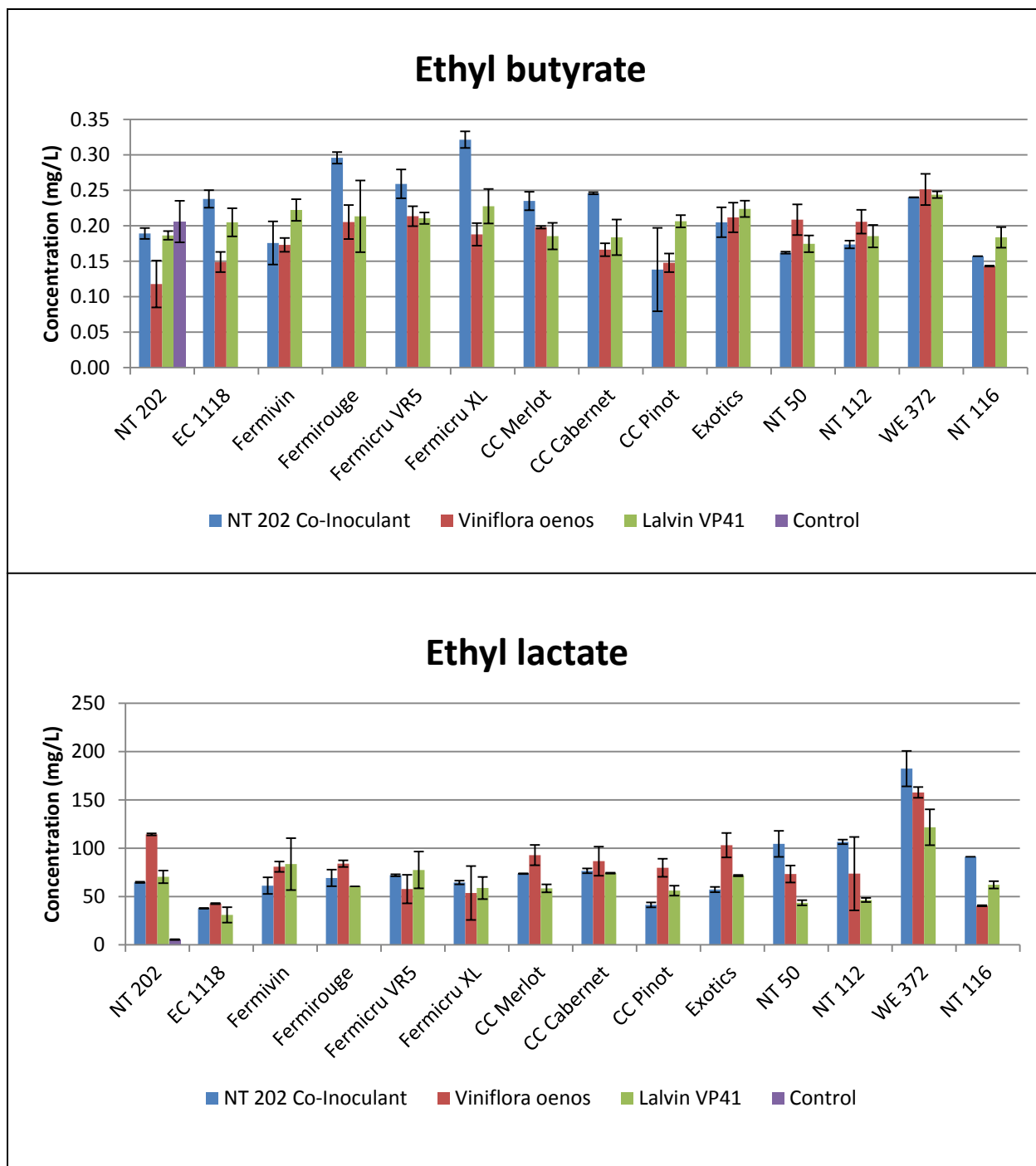


Figure 3.15 Ethyl butyrate and ethyl lactate production (mg/L) observed in Merlot 2011 vintage after completion of co-inoculated MLF. Concentrations represent the average of duplicate treatments, each determined in duplicate by GC-FID.

Malolactic fermentation resulted in higher total esters compared to the un-inoculated MLF control (**Figure 3.16**). This is in accordance with a previous study on co-inoculation on Pinotage by Lerm (2010) and another study on sequential MLF in Pinotage and Shiraz by Malherbe (2010). Increased levels of total esters might be attributed to difference in esterase activity between the LAB- and yeast strains. The highest total ester production was observed for yeast treatment WE 372 and the lowest for EC1118, irrespective of MLF starter culture used during co-inoculation. For the majority of the yeast treatments (including WE 372), *Viniflora oenos* resulted in higher total esters than *Lalvin VP41*. In contrast to this, Malherbe (2010) found that *Lalvin VP41* produced higher total esters than *Viniflora oenos* when used in sequential inoculation with WE 372. In general no discernible trends were visible between MLF treatments with regard to ester production.

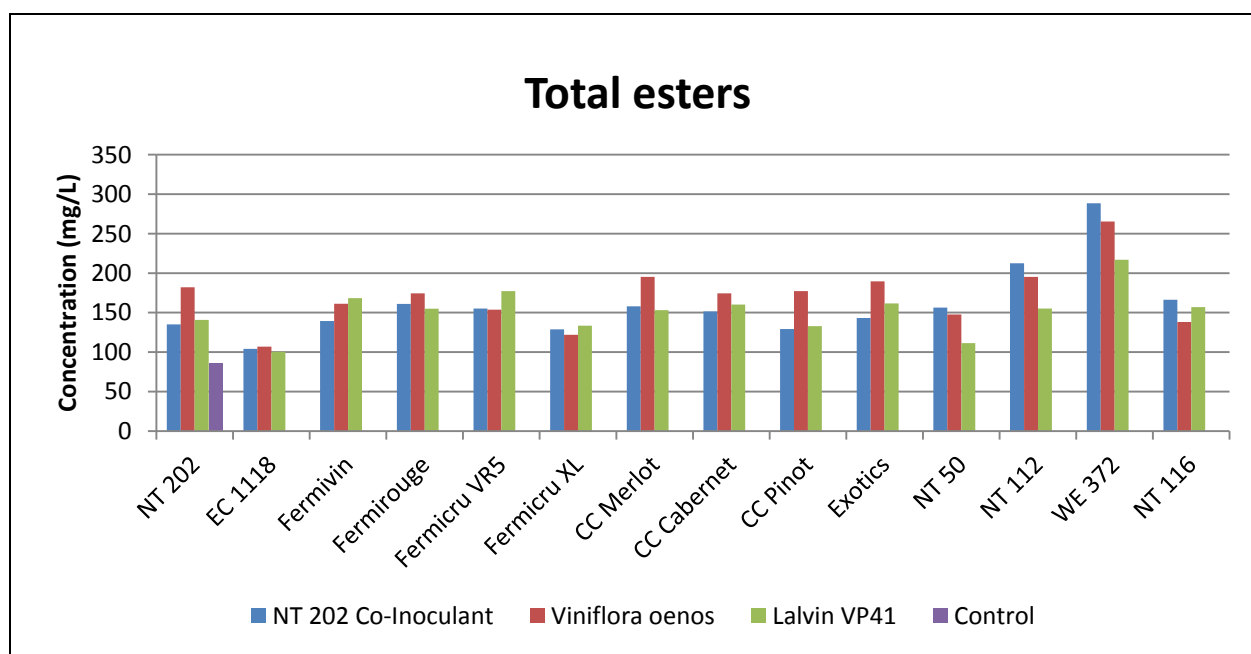


Figure 3.16 The total ester production (mg/L) observed in Merlot 2011 vintage after completion of co-inoculated MLF. Concentrations represent the average of duplicate treatments.

Volatile fatty acids

Acetic acid is both quantitatively and sensorially the most important volatile acid produced during vinification. In concentrations exceeding 0.7 g/L (Swiegers *et al.*, 2005), acetic acid leads to vinegary, pungent aromas in wine (Francis and Newton, 2005). Lower concentrations (0.2 to 0.6 g/L) can contribute to the complexity of wine aroma (Lerm *et al.*, 2010). The 2011 Merlot results support previous findings by Lerm (2010) using co-inoculation that acetic acid was

quantitatively the predominant acid produced. The highest and lowest concentrations of acetic acid were observed for Fermirouge and Exotics, respectively, regardless of the MLF starter culture used (**Tables 3.10** and **3.12**). NT 112 and WE 372 showed the highest and Fermicru XL the lowest concentrations of propionic acid (pungent, rancid, sweat) (Francis and Newton, 2005), irrespective of the MLF starter culture used. For isobutyric acid (rancid, butter, cheese) (Francis and Newton, 2005), NT 202, CC Merlot, NT 50, NT 112, WE 372 and NT 116 showed lower concentrations of isobutyric acid whereas the highest concentration was observed for Fermirouge, regardless of MLF starter culture used. For yeast NT 202, similar concentrations, in isobutyric acid, were observed, regardless of the MLF treatment. For butyric acid (cheese) (Escudero *et al.*, 2007), the lowest concentrations were observed for NT 202, CC Pinot and NT 116. EC1118 and CC Pinot showed lower concentrations of isovaleric acid (cheese) (Escudero *et al.*, 2007), compared to the un-inoculated MLF control. Although valeric acid was detected in low concentrations, the lowest concentrations were observed for EC1118, Fermivin, Fermicru VR5, Fermicru XL, CC Merlot and NT 50. The same trend in production of hexanoic acid (sweat, rancid cheese, fatty) (Peinado *et al.*, 2004; Francis and Newton, 2005) were observed for yeast treatments, regardless of the MLF starter culture used, of which WE 372 showed the lowest concentrations. A study done by Maicas *et al.* (1999) that found no significant increase in isobutyric or hexanoic acids after completion of MLF, support these findings. Decanoic acid, if detected, was detected in small quantities only for some yeast treatments.

By comparing the total volatile fatty acid concentrations observed for the un-inoculated MLF control with the rest of the treatments, a general increase in total volatile fatty acids was evident (**Figure 3.17**). The highest total volatile fatty acid concentrations were observed for Fermirouge (when co-inoculated with NT 202 Co-Inoculant and *Viniflora oenos*) and Fermicru VR5 (when co-inoculated with *Lalvin VP41*). An overall trend of increased total volatile fatty acids concentrations were observed for the different MLF treatments, compared to the un-inoculated MLF control. The NT 202 Co-Inoculant showed lower concentrations of total volatile fatty acids for all yeast treatments, compared to the *O. oeni* MLF starter cultures *Viniflora oenos* and *Lalvin VP41*. This might be due to the presence of *L. plantarum* in the mixed NT 202 Co-Inoculant starter culture that might affect the formation of volatile fatty acids. Due to the low detection threshold and undesired pungent aromas of many of the volatile fatty acids, lower production of these compounds is desired rather than higher concentrations (Francis and Newton, 2005).

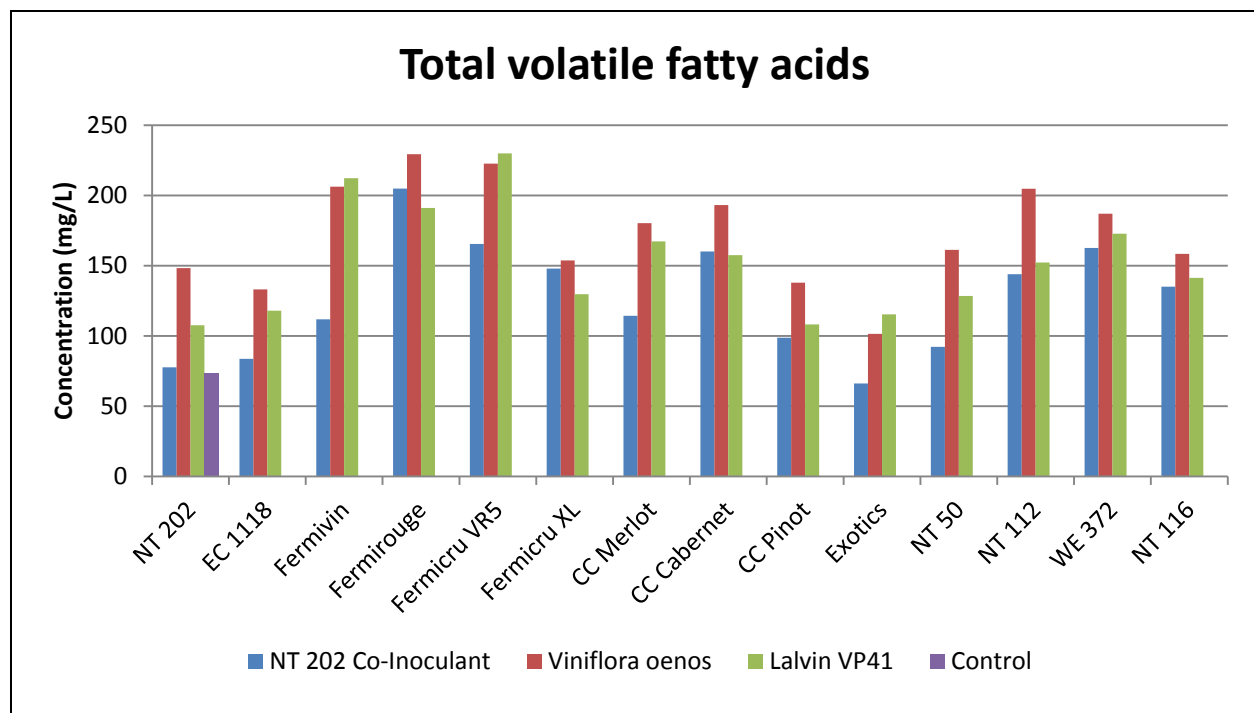


Figure 3.17 The total volatile fatty acid production (mg/L) observed in Merlot 2011 vintage during co-inoculation. Concentrations represent the average of duplicate treatments.

Higher alcohols

Higher alcohol concentrations have either a positive or negative impact on wine aroma. At higher concentrations (more than 400 mg/L), higher alcohols could be unfavourable to wine aroma due to harsh, chemical-like aromas, whereas lower concentrations (less than 300 mg/L) can contribute to the complexity and fruity aromas in wine (Swiegers *et al.*, 2005). Results show (Tables 3.10 to 3.12) that all higher alcohols, except isoamyl alcohol, were present in concentrations below 300 mg/L indicating that most of the higher alcohols production had a beneficial impact on wine aroma. Results showed a general increase in isobutanol, isoamyl alcohol and 2-phenylethanol as a result of MLF. This was also found by Malherbe (2010), but a study done by Herjavec *et al.* (2001) found no change in the aforementioned compounds. Methanol and isoamyl alcohol were quantitatively the predominant higher alcohols after completion of co-inoculated MLF in the 2011 Merlot. This is in agreement with results found in a study by Lerm (2010) and De Revel *et al.* (1999). Results showed that MLF led to increased levels of isobutanol, isoamyl alcohol and 2-phenylethanol.

The lowest concentration of methanol was observed for Fermirouge. Propanol (ripe fruit, alcohol) and 3-ethoxy-1-propanol (fruity) (Peinado *et al.*, 2004) exhibited similar trends in production where the highest concentrations were observed for yeast treatment NT 112 (**Figure 3.18**).

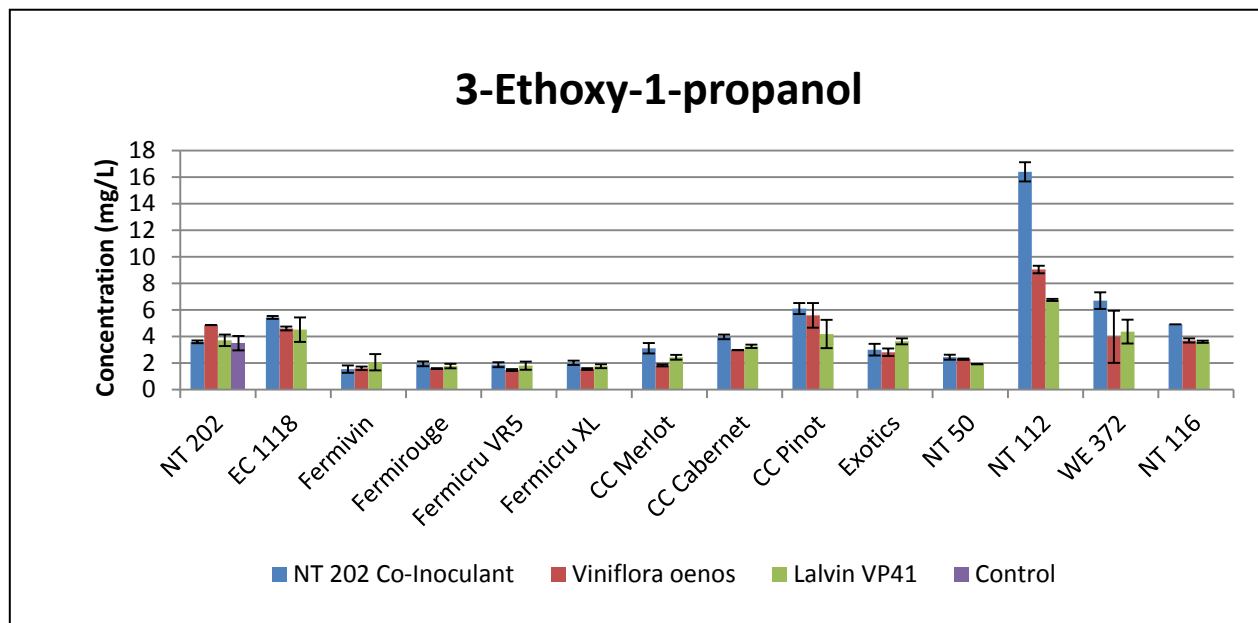


Figure 3.18 3-Ethoxy-1-propanol production (mg/L) observed in the 2011 Merlot after completion of co-inoculated MLF. Concentrations represent the average of duplicate treatments, each determined in duplicate by GC-FID.

NT 202 showed the lowest concentration of isobutanol (alcohol, solvent) (Peinado *et al.*, 2004), whereas Fermirouge and CC Pinot showed the highest concentrations. For butanol (alcohol, solvent) (Peinado *et al.*, 2004) the lowest concentrations were observed for Fermivin, Fermirouge, Fermicru VR5, Fermicru XL and CC Merlot. The highest concentrations of butanol were observed for CC Cabernet and Exotics. In general, it seems that MLF leads to increased concentrations of isoamyl alcohol (fusel, alcohol) (Peinado *et al.*, 2004; Escudero *et al.*, 2007). The highest concentrations of isoamyl alcohol were observed for CC Pinot and Exotics whereas the lowest concentration was observed for Fermirouge, when co-inoculated with Lalvin VP41. For most yeast treatments, 4-methyl-1-pentanol showed increased levels as a result of MLF. 4-Methyl-1-pentanol and 3-methyl-1-pentanol also exhibited similar production trends; Exotics showed the highest concentrations, whereas NT 112 and NT 116 showed the lowest concentrations of these compounds. For hexanol (rose) (Peinado *et al.*, 2004), no discernible trends between MLF treatments were apparent, except that MLF led to increased concentrations. Varying concentrations were observed for the different yeast treatments with

regard to hexanol production. This is in contrast to a study done by Ugliano and Moio (2005) that found a general negligible impact of MLF on higher alcohol production, except for an increase in hexanol and 3-methyl-1-pentanol. A general increase in 2-phenylethanol (rose) (Peinado *et al.*, 2004) was observed between the un-inoculated MLF treatment and the remaining co-inoculated treatments of which yeast treatments CC Cabernet, CC Pinot and Exotics showed higher concentrations than the rest.

Higher concentrations of total higher alcohols were observed for NT 202 in combination with *Viniflora oenos* and *Lalvin VP41* compared to NT 202 Co-Inoculant or the un-inoculated MLF control (**Figure 3.19**). The highest concentration of total higher alcohols was observed for yeast CC Pinot in combination with NT 202 Co-Inoculant while the lowest concentration was observed for *Fermirouge* in combination with *Lalvin VP41*. For yeast strains *Fermicru VR5*, *Fermicru XL*, CC Cabernet and CC Pinot total higher alcohol concentrations observed were higher in combination with NT 202 Co-Inoculant, followed by co-inoculation with *Lalvin VP41* and then *Viniflora oenos*. In general MLF seems to increase the concentration of total higher alcohols compared to the un-inoculated control. This is in contrast to results obtained by other studies including De Revel *et al.* (1999), Pozo-Bayón *et al.* (2005), Ugliano and Moio (2005) and Jeromel *et al.* (2008) where it was found that MLF had an insignificant effect on higher alcohol production, except for an increase in isobutanol and 2-phenylethanol (Jeromel *et al.*, 2008), an increase in hexanol and 3-methyl-1-pentanol (Ugliano and Moio, 2005) and an increase in isoamyl alcohol (De Revel *et al.*, 1999). Generally production of propanol, isobutanol, butanol, isoamyl alcohol, 4-methyl-1-pentanol, 3-methyl-1-pentanol, 3-ethoxy-1-propanol and 2-phenylethanol were found to be dependent on yeast strain rather than LAB strain. This is in contrast with results from Malherbe (2010) and Maicas *et al.* (1999). Malherbe (2010) found that the production of the aforementioned compounds, except for 4-methyl-1-propanol, were LAB strain dependent. Maicas *et al.* (1999) found that propanol, isobutanol, butanol and isoamyl alcohol were strain dependent.

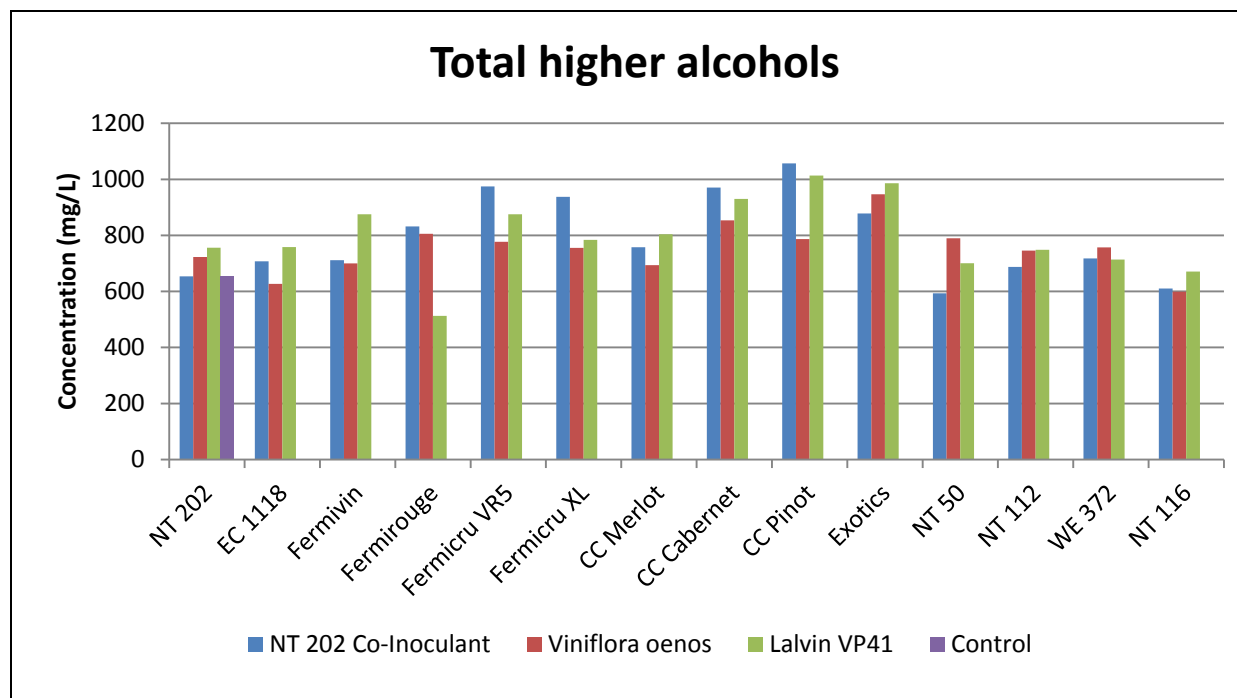


Figure 3.19 The total higher alcohol production (mg/L) observed in Merlot 2011 vintage during co-inoculation. Concentrations represent the average of duplicate treatments.

Monoterpenes

It has been proven that various LAB possess β -glucosidase activity that can liberate glycosidically bound aroma precursors present in the grapes to form aromatically active aroma compounds such as monoterpenes (Matthews *et al.*, 2004; Sestelo *et al.*, 2004). The 2011 Merlot wines that were subjected to GC-FID analyses for monoterpenes included all the NT 202 Co-Inoculant treated wines as well as the un-inoculated MLF control, but only the NT 202 yeast treatments with *Viniflora oenos* and *Lalvin VP41* (**Table 3.13**). Of the 15 monoterpenes that were monitored, only α -terpeneol, citronellol, nerol and geraniol were detected (**Figure 3.20**). α -Terpeneol (lilac) (Knoll *et al.*, 2011) was only detected for yeast treatments CC Pinot, NT 112, WE 372 and NT 116, in co-inoculation with NT 202 Co-Inoculant, but not for yeast NT 202 when co-inoculated with *Viniflora oenos* or *Lalvin VP41* or un-inoculated. The highest concentration of α -terpeneol was observed for the un-inoculated MLF control indicating that MLF leads to lower concentrations of this compound in the final wine. This is in contrast to a study done by Knoll *et al.* (2012) that found increased levels of α -terpeneol after MLF. The highest concentration for citronellol (citrus) (De Klerk, 2008) was observed for EC1118, co-inoculated with NT 202 Co-

Inoculant, compared to the un-inoculated MLF control and the remaining *O. oeni* MLF starter cultures. The lowest concentration was observed for CC Merlot, co-inoculated with NT 202 Co-Inoculant. Yeast treatments Fermivin, Fermirouge, Fermicru VR5, Fermicru XL, CC Merlot, Exotics, NT 112 and WE 372, co-inoculated with NT 202 Co-Inoculant, produced lower concentrations of citronellol than the un-inoculated control. The remaining yeast treatments (except for EC1118) produced similar concentrations of citronellol compared to the un-inoculated control indicating that interaction between the LAB and the specific yeast did not affect citronellol concentrations. Malolactic fermentation resulted in higher concentrations of nerol for yeast strains CC Cabernet, CC Pinot, Exotics and NT 50, co-inoculated with NT 202 Co-Inoculant, compared to the un-inoculated MLF control and the remaining yeast treatments. The highest nerol concentrations were observed for yeast treatments CC Cabernet and CC Pinot, co-inoculated with NT 202 Co-Inoculant. For yeast strain NT 202, similar concentrations in nerol concentrations were observed, regardless of the MLF starter culture used. The lowest nerol concentration observed for the 2011 Merlot, was the treatment where CC Merlot were co-inoculated with NT 202 Co-Inoculant. The highest concentrations of geraniol (rose, geranium) (Francis and Newton, 2005) were observed for yeast treatments Fermicru XL, Exotics and WE 372, co-inoculated with NT 202 Co-Inoculant, in similar concentrations. For NT 202, the highest geraniol concentration was observed for the un-inoculated MLF control, followed by co-inoculation with *Viniflora oenos*, Lalvin VP41 and NT 202 Co-Inoculant. The latter treatment is also the lowest geraniol-producing treatment.

The highest total monoterpene concentration was observed for yeast WE 372 and the lowest concentration was observed for yeast NT 202, used in co-inoculation with NT 202 Co-Inoculant. From the results it is evident that the interaction between NT 202 and the NT 202 Co-Inoculant, containing the *L. plantarum* strain, leads to a lower concentration of total monoterpenes after co-inoculated MLF compared to the *O. oeni* cultures *Viniflora oenos* and Lalvin VP41. Although both *L. plantarum* and *O. oeni* have been proven to have β -glucosidase activity, possible interaction between the two LAB strains might alter their enzymatic activity leading to lower production of monoterpenes (Sestelo *et al.*, 2004; Grimaldi *et al.*, 2005a, b).

Table 3.13 Monoterpenes ($\mu\text{g/L}$) measured in 2011 in Merlot after completion of co-inoculated MLF. Concentrations represent the average of duplicate treatments (standard deviations not shown), each analysed by GC-FID (nd: not detected).

Monoterpenes	NT 202 Co-Inoculant															Viniflora oenos	Lalvin VP41	Control
	NT 202	EC-1118	Fermivin	Fermirouge	Fermicru VR5	Fermicru XL	CC Merlot	CC Cabernet	CC Pinot	Exotics	NT 50	NT 112	WE 372	NT 116	NT 202	NT 202	NT 202	
α -Terpeneol	nd	nd	nd	nd	nd	nd	nd	nd	0.83	nd	nd	0.94	0.88	1.06	0.86	0.99	1.10	
Citronellol	9.12	11.40	5.53	4.44	5.95	5.67	1.98	8.81	10.11	5.12	8.84	5.77	2.73	9.25	10.20	8.97	7.68	
Nerol	6.35	6.54	5.96	6.09	6.52	6.54	4.35	10.35	10.48	7.19	7.66	5.25	4.67	5.12	6.05	5.77	5.49	
Geraniol	71.39	134.60	279.43	313.06	229.36	385.37	334.13	286.77	224.82	372.46	220.92	252.64	418.03	238.80	220.25	195.15	252.91	
Total Monoterpenes	86.86	152.54	290.92	323.59	241.83	397.58	340.46	305.93	246.24	384.77	237.42	264.60	426.30	254.23	237.36	210.89	267.18	

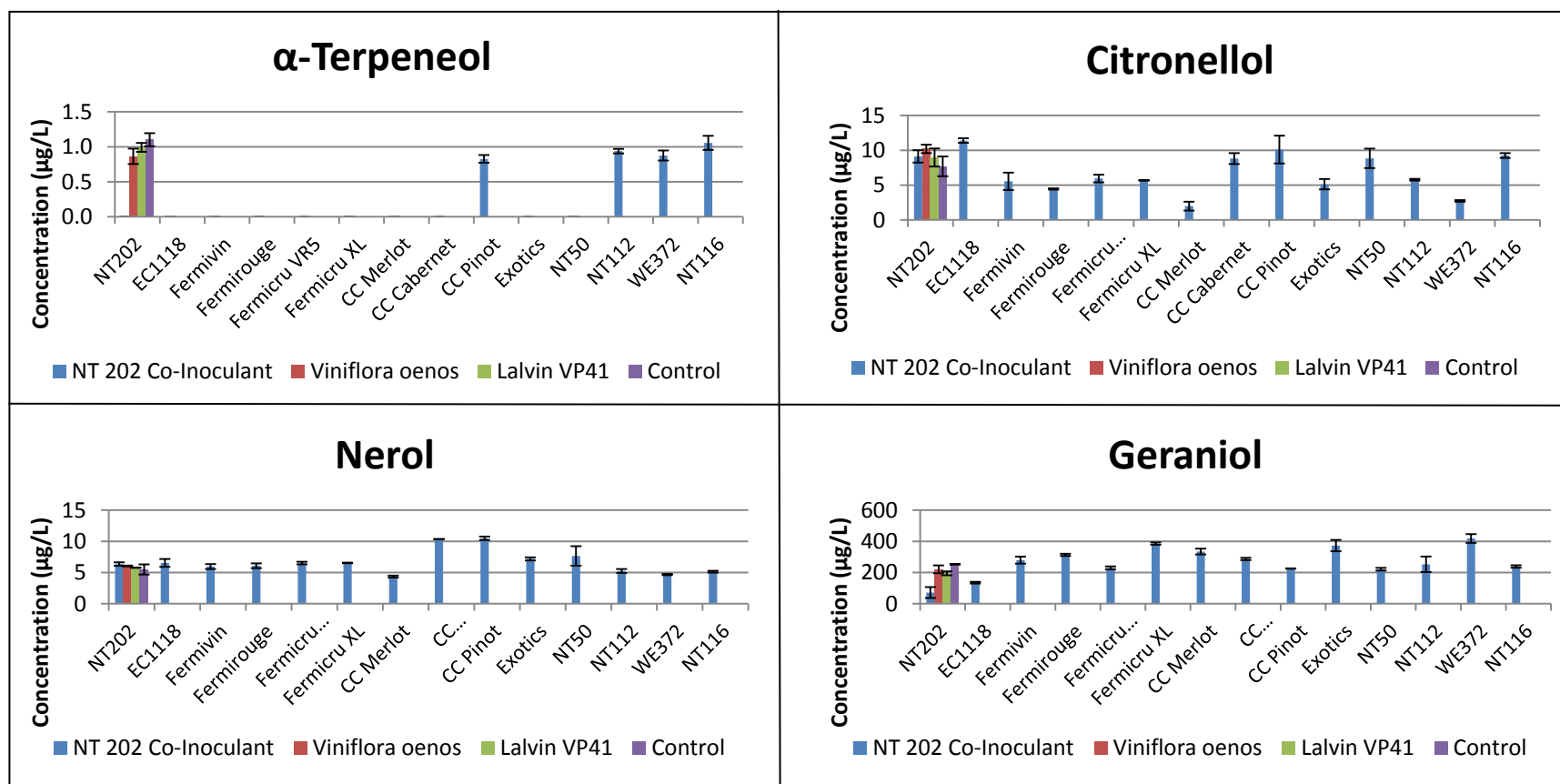


Figure 3.20 Monoterpene production ($\mu\text{g/L}$) observed in the 2011 Merlot after completion of co-inoculated MLF. Values represent averages of duplicate treatments.

Carbonyl Compounds

Results from the principal carbonyl compound analyses done on the 2011 Merlot can be seen in **Table 3.14** and **Figure 3.21**. Diacetyl (2,3-butanedione) is the most important carbonyl compound associated with MLF since it contributes to the buttery, nutty and butterscotch aromas in the wine (Bartowsky and Henschke, 2004). The highest diacetyl concentrations were observed for yeast treatments EC1118, NT 50 and NT 116, co-inoculated with NT 202 Co-Inoculant, as well as NT 202, co-inoculated with *Viniflora oenos*. All treatments showed increased diacetyl concentrations after completion of MLF compared to the un-inoculated MLF control. This trend was also observed by Lerm (2010) in Pinotage, Cabernet Sauvignon and Chardonnay during co-inoculation as well as sequential inoculation. This can be attributed to the presence of LAB in all treatments, except the un-inoculated control. Lactic acid bacteria, present during MLF, metabolize citric acid to form diacetyl as an intermediate (Bartowsky and Henschke, 2004). Concentrations of 2,3-pentadione (butter, cream) (Escudero *et al.*, 2007) were lower for the the *O. oeni* MLF starter cultures, compared to the un-inoculated control and the NT 202 Co-Inoculant treatments. This indicated that the presence of *L. plantarum* in the starter culture lead to increased concentrations of 2,3-pentadione. The highest concentration of 2,3-pentadione were observed for Exotics, whereas the lowest concentration was observed for NT 202, when co-inoculated with *Viniflora oenos*. The highest concentration of acetoin (butter, cream) (Francis and Newton, 2005) production was observed for yeast strains NT 50 and EC1118, co-inoculated with NT 202 Co-Inoculant, whereas the lowest concentration was observed for the un-inoculated control.

Trends of the total carbonyl compounds mimic that of acetoin due to its quantitative predominance. Malolactic fermentation showed a clear increase in total carbonyl compounds, especially diacetyl and acetoin, compared to the un-inoculated MLF control. This is due to citric acid metabolism, as previously mentioned, where the chemically unstable diacetyl is reduced to acetoin, which then also explains why acetoin levels are higher than diacetyl levels (Lerm *et al.*, 2010). It is interesting to note that two of the yeast treatments that were classified as neutral towards MLF (EC1118 and NT 50), due to their slower MLF rate (**Table 3.8**), produced the highest diacetyl concentrations. This phenomenon has been mentioned before by Swiegers *et al.* (2005).

Table 3.14 Principal carbonyl compounds (mg/L) measured in the 2011 Merlot after completion of co-inoculated MLF. Values represent duplicate treatments (standard deviations not shown), analysed using GC-MS.

	NT 202 Co-Inoculant															Viniflora oenos	Lalvin VP41	Control
	NT202	EC1118	Fermivin	Fermirouge	Fermicru VR5	Fermicru XL	CC Merlot	CC Cabernet	CC Pinot	Exotics	NT50	NT112	WE372	NT116	NT202	NT202	NT202	
Diacetyl	10.682	14.800	9.427	7.022	10.205	9.875	11.663	10.527	8.803	7.537	14.163	10.868	10.050	13.507	14.211	11.058	2.314	
2,3-pentadione	1.054	1.055	1.103	0.767	1.182	0.980	0.964	1.051	0.883	1.238	0.947	0.750	0.869	0.882	0.576	0.629	1.089	
Acetoin	10.966	22.836	5.352	4.673	5.407	6.478	5.853	8.799	18.550	13.380	31.027	9.779	8.425	17.969	15.426	12.427	3.954	
Total carbonyl compounds	22.702	38.691	15.883	12.462	16.793	17.333	18.480	20.376	28.236	22.155	46.136	21.397	19.343	32.359	30.213	24.114	7.357	

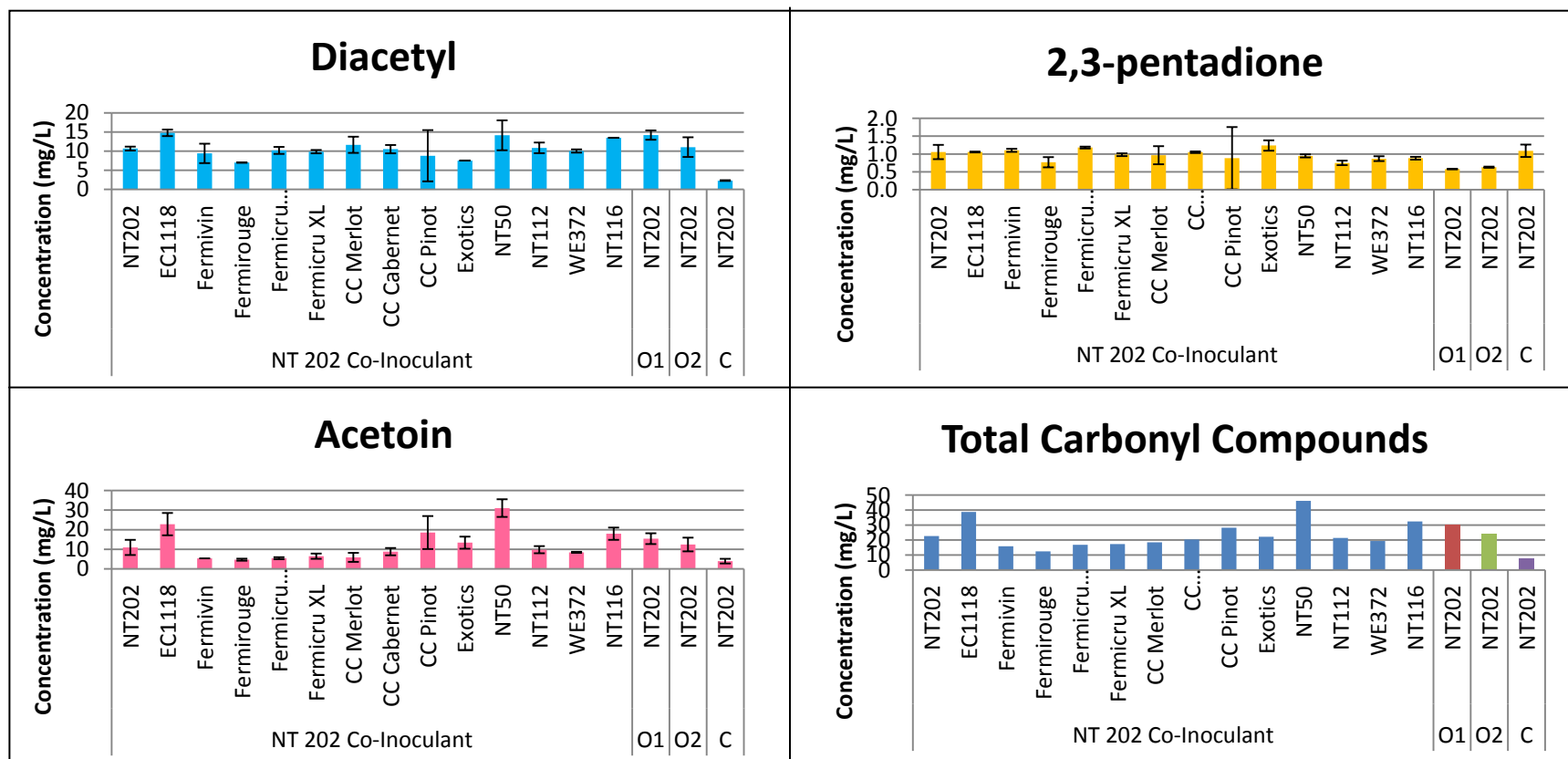


Figure 3.21 Principal carbonyl compounds (mg/L) measured in the 2011 Merlot after completion of co-inoculated MLF. O1, O2 and C represent Viniflora oenos, Lalvin VP41 and the un-inoculated MLF control respectively. Values represent treatments in duplicate as analysed by GC-MS.

Principal component analysis (PCA) was conducted on the GC-FID and GC-MS generated data of the 2011 Merlot wines to investigate correlations between different treatment samples, co-inoculated with NT 202 Co-Inoculant, and aroma attributes (**Figure 3.22**). The bi-plot shows the correlation between the samples (objects) and attributes (variables) concurrently. Scores, presented in blue, indicate the different treatments, co-inoculated with NT 202 Co-Inoculant, and the loadings, presented in red, indicate the aroma compounds. Scores and loadings that are close to each other are positively correlated. Loadings close to a score indicate which variables mostly influence the sample by means of positive correlation.

WE 372 were highly correlated with ethyl lactate (supported by results obtained for ethyl lactate production in **Figure 3.15**) and ethyl acetate. As expected, diacetyl and acetoin was highly correlated, but unexpectedly correlated to the un-inoculated MLF control across PC2, together with yeast treatments NT 202 and EC1118. This might indicate that another factor is driving the correlation. The correlation between EC1118 and acetoin are supported by carbonyl compound analysis results as seen in **Figure 3.21**.

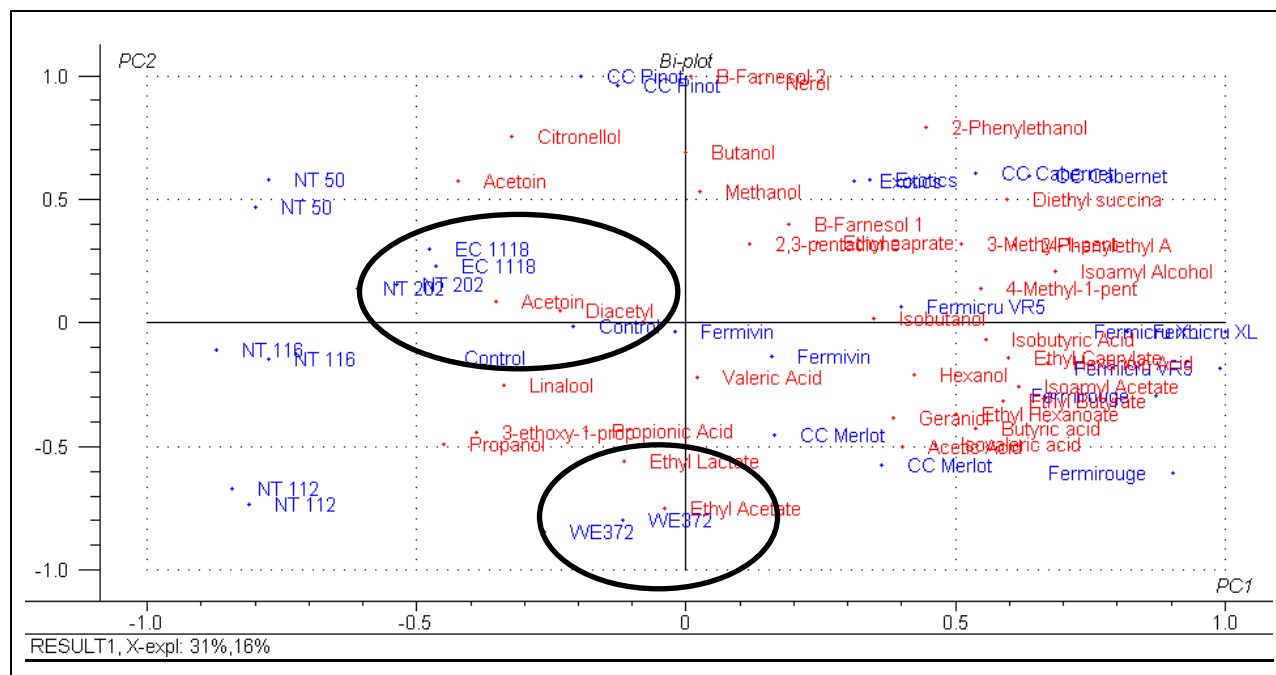


Figure 3.22 PCA bi-plot of scores (in blue) and loadings (in red) of the GC-FID and GC-MS generated data for the 2011 Merlot after completion of co-inoculated MLF using NT 202 Co-Inoculant. NT 202 yeast was used for the un-inoculated MLF control.

2012 Shiraz

The GC-generated results of the 2012 Shiraz were used to determine the changes in volatile composition that could be attributed to the different yeast/MLF starter culture combinations and to study trends in the volatile profile of the wines.

Impact of the yeast-bacterial combinations on aroma compound production

The major volatiles (esters, higher alcohols and volatile fatty acids) that were detected in the 2012 Shiraz for determining the impact of the yeast-bacterial combinations on the aroma compound production are listed in **Table 3.15**. No discernible differences were observed for ethyl acetate and decanoic acid and will therefore not be discussed. Similar trends in observation of the following compounds were observed: isoamyl acetate, ethyl hexanoate, ethyl caprylate, isoamyl alcohol, 3-methyl-1-pentanol (**Figure 3.25**), isobutyric acid, isovaleric acid, valeric acid and hexanoic acid. For all the aforementioned compounds, Exotics and WE 372 showed higher concentrations compared to the remaining treatments. This supports the observations of the Merlot 2011 vintage that found that WE 372 showed the highest concentrations of various esters.

Esters

The highest concentration of ethyl butyrate was observed for WE 372 followed by Exotics, NT 202 and NT 50. Small variations between treatments were observed for the following esters: hexyl acetate and 2-phenylethyl acetate. Malolactic fermentation showed a clear increase in ethyl lactate concentrations for all treatments (**Figure 3.23**). This supported the findings of the previous vintage and other studies previously mentioned. NT 50 showed the highest concentration of ethyl lactate, followed by WE 372, NT 202 and Exotics. As previously mentioned, this increase was expected due to the increased lactic acid concentrations caused by MLF and the presence of ethanol, which reacts with the lactic acid to form ethyl lactate. If present, ethyl caprate was detected in small quantities, except for the un-inoculated MLF control. For diethyl succinate, the highest concentrations were observed for WE 372 (**Figure 3.23**). Results showed a general trend that co-inoculation with the mixed MLF starter culture NT 202 Co-Inoculant led to higher diethyl succinate concentrations than for Lalvin VP41, regardless of the yeast strain used. This supports the findings of the Merlot 2011 vintage. This is in contrast to the findings of Lerm (2010) where co-inoculation with Lalvin VP41 led to higher diethyl

succinate concentrations than mixed MLF starter cultures containing *O. oeni* as well as *L. plantarum*. The highest concentration of 2-phenylethyl acetate was observed for Exotics, whereas the remaining treatments showed no discernible differences in concentration.

Table 3.15 Esters, higher alcohols and volatile fatty acids (mg/L) measured in the 2012 Shiraz after completion of co-inoculated MLF using NT 202 Co-Inoculant and Lalvin VP41 for determining the impact of the yeast-bacterial combinations on the aroma compound production. Concentrations represent the average of triplicate treatments (standard deviations not shown), each analysed in duplicate by GC-FID (nd: not detected).

Esters	NT 202 Co-Inoculant				Lalvin VP41				Control
	Exotics	NT 50	WE 372	NT 202	Exotics	NT 50	WE 372	NT 202	NT 202
Ethyl Acetate	149.30	154.08	140.71	140.42	134.76	144.54	144.57	146.36	161.71
Ethyl Butyrate	0.56	0.41	0.64	0.47	0.51	0.40	0.58	0.49	0.51
Isoamyl Acetate	6.57	4.35	6.59	5.04	5.30	4.19	5.82	4.99	5.41
Ethyl Hexanoate	0.64	0.49	0.63	0.49	0.59	0.50	0.59	0.48	0.51
Hexyl Acetate	0.04	0.04	0.06	0.05	0.03	0.05	0.05	0.06	0.06
Ethyl Lactate	35.49	49.47	46.53	41.56	33.31	59.62	47.70	38.39	7.63
Ethyl Caprylate	0.21	0.17	0.20	0.17	0.20	0.17	0.19	0.15	0.17
Ethyl caprate	nd	nd	0.05	0.04	nd	nd	0.06	0.05	1.66
Diethyl succinate	0.55	0.49	0.77	0.58	0.46	0.42	0.66	0.48	0.52
2-Phenylethyl Acetate	0.71	0.59	0.56	0.54	0.65	0.59	0.54	0.54	0.58
Total esters	194.07	210.10	196.75	189.37	175.82	210.47	200.76	191.98	178.76
Higher alcohols									
Methanol	285.95	288.68	205.76	208.69	296.72	256.81	209.20	244.02	236.39
Propanol	141.69	133.38	230.66	192.55	149.09	127.24	236.01	205.06	178.79
Isobutanol	56.60	53.05	48.18	38.74	60.09	53.83	51.21	38.55	37.18
Butanol	8.34	4.88	4.93	4.63	8.53	4.67	4.87	4.72	4.18
Isoamyl Alcohol	485.04	360.50	446.82	339.68	487.90	346.10	454.88	343.21	333.41
4-Methyl-1-pentanol	0.02	nd	0.03	nd	0.02	nd	0.03	nd	nd
3-Methyl-1-pentanol	0.34	0.15	0.31	0.19	0.32	0.14	0.30	0.17	0.19
Hexanol	3.04	3.12	3.18	3.01	3.29	2.90	3.17	3.18	3.26
3-ethoxy-1-propanol	3.65	3.25	9.98	6.07	3.98	3.24	11.57	6.52	5.06
2-Phenylethanol	97.26	65.72	63.89	51.16	102.84	64.57	67.77	52.31	48.45
Total higher alcohols	1081.92	912.72	1013.76	844.72	1112.78	859.48	1039.00	897.75	846.90
Volatile fatty acids									
Acetic acid	236.33	446.29	485.48	372.70	276.16	655.42	535.30	372.50	376.12
Propionic Acid	2.91	3.91	5.54	4.94	3.51	4.68	6.06	4.50	7.34
Isobutyric Acid	2.82	1.95	2.70	1.92	3.05	2.26	3.12	1.88	2.21
Butyric acid	2.10	1.81	2.23	1.66	2.16	1.78	2.35	1.66	2.80
Isovaleric acid	2.69	1.95	2.40	2.21	2.92	2.17	2.66	2.55	2.89
Valeric Acid	0.55	0.45	0.79	0.53	0.68	0.44	0.85	0.54	0.50
Hexanoic Acid	3.01	2.38	2.83	2.03	2.85	2.29	2.84	2.04	2.62
Decanoic Acid	0.67	0.59	0.66	0.59	0.57	0.64	0.67	0.57	0.56
Total volatile fatty acids	251.08	459.33	502.63	386.59	291.90	669.67	553.85	386.25	395.04

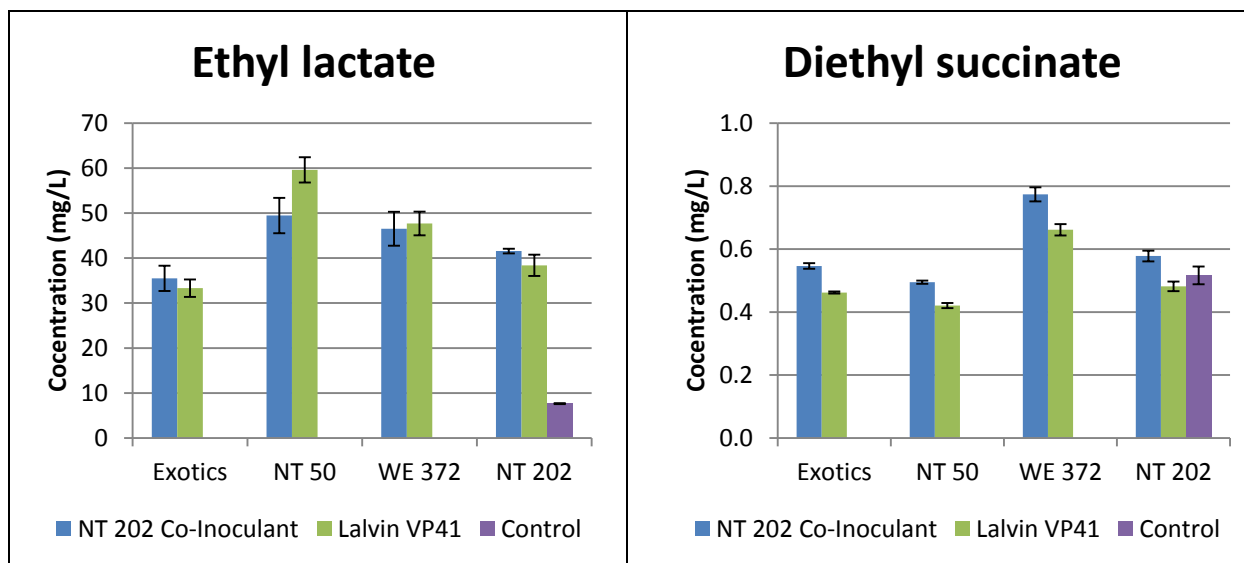


Figure 3.23 Esters (mg/L) measured after completion of MLF for determining the impact of the yeast-bacterial combinations on the aroma compound production in the 2012 Shiraz. Values represent triplicate treatments as analysed using GC-FID.

Malolactic fermentation resulted in higher total esters compared to the un-inoculated MLF control (**Figure 3.24**). This supports observations of the Merlot 2011 vintage and can, as before, be attributed to difference in esterase activity between the LAB- and yeast strains. NT 50 showed the highest total ester concentration implying that wines of this treatment will probably be perceived as more fruity than the other treatment wines.

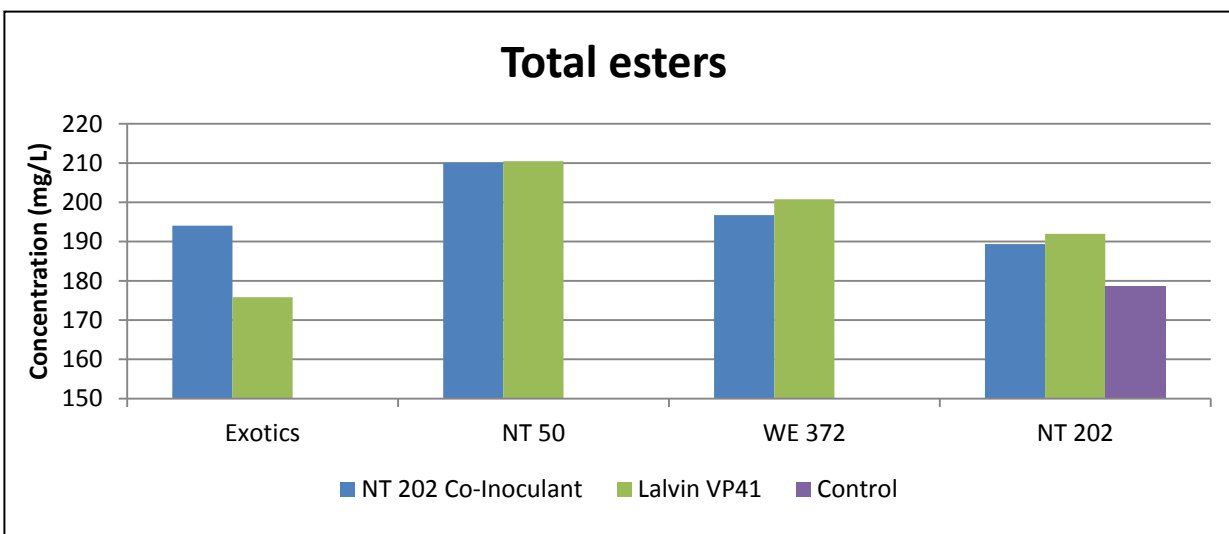


Figure 3.24 Total esters (mg/L) observed in the 2012 Shiraz for determining the impact of the yeast-bacterial combinations on the aroma compound production after completion of co-inoculated MLF. Values represent averages of triplicate treatments, analysed by GC-FID.

Higher alcohols

As with the 2011 Merlot and other studies mentioned, isoamyl alcohol was quantitatively the predominant higher alcohol observed and also the only higher alcohol to show concentrations above 300 mg/L which could have an undesirable effect on wine aroma. Exotics and NT 50 produced higher concentrations of methanol, compared to the un-inoculated MLF control, whereas the opposite trend was observed for propanol whereby WE 372 and NT 202 produced higher concentrations of propanol, compared to the un-inoculated MLF control (**Table 3.15**). For isobutanol all treatments, except NT 202, produced higher concentrations compared to the un-inoculated MLF control. Exotics was the only yeast treatment that showed increased concentrations of butanol after completion of MLF, compared to the un-inoculated MLF control (**Figure 3.25**). Isoamyl alcohol showed increased levels after completion of MLF. This is in accordance with results obtained for the Merlot 2011 vintage as well as other studies mentioned. Isoamyl alcohol showed similar trends in production as 3-methyl-1-pentanol as previously discussed. 4-Methyl-1-pentanol was only detected for Exotics, WE 372 and the un-inoculated MLF control (in small quantities). No discernible differences were observed between treatments for hexanol. WE 372 and NT 202 were the only treatments that showed increased concentrations of 3-ethoxy-1-propanol compared to the un-inoculated control, of which Exotics showed the highest levels of production (**Figure 3.25**). This is in agreement with results obtained for the 2011 Merlot, which indicated that production of 3-ethoxy-1-propanol was yeast strain dependent. Exotics, followed by NT 202 and WE 372 (in similar quantities) showed higher concentrations of 2-phenylethanol compared to the un-inoculated MLF control (which showed similar concentrations as for NT 202) (**Figure 3.25**).

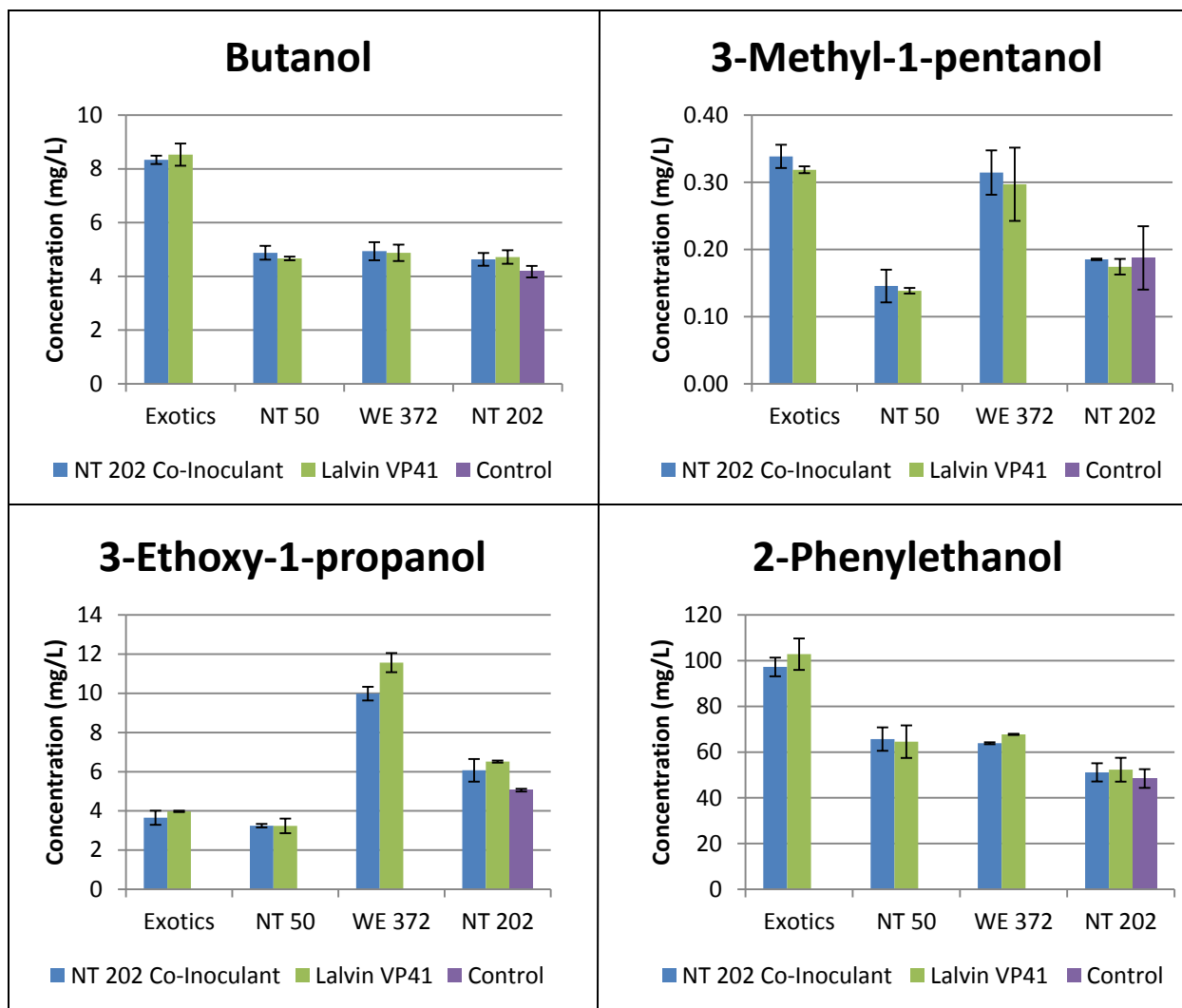


Figure 3.25 Higher alcohols (mg/L) measured after completion of co-inoculated MLF for determining the impact of the yeast-bacterial combinations on the aroma compound production in the 2012 Shiraz. Values represent triplicate treatments as analysed using GC-FID.

The highest total higher alcohol concentration was observed for Exotics followed by WE 372, NT 50 and NT 202 (**Figure 3.26**). Although most of the higher alcohols were produced at concentrations lower than 300 mg/L which may contribute positively to wine aroma, the lower production of these higher alcohols (such as for NT 202) could be beneficial for wine aroma. At high concentrations these alcohols may impart harsh, solvent- or chemical-like aroma to the wine.

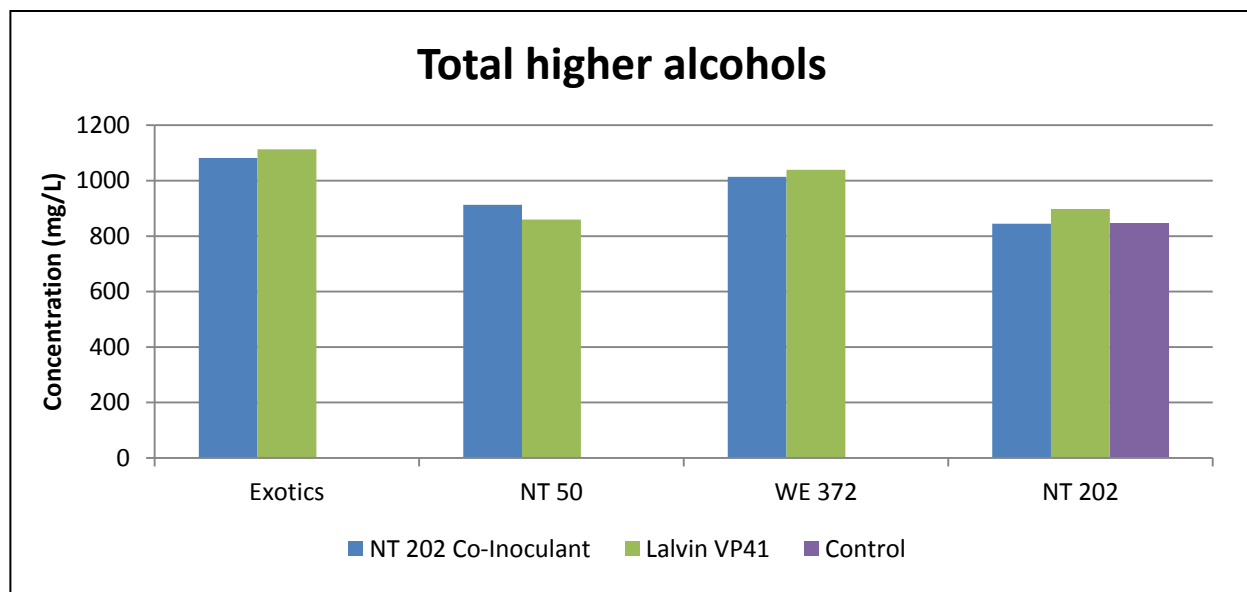


Figure 3.26 Total higher alcohols (mg/L) observed in the 2012 Shiraz for determining the impact of the yeast-bacterial combinations on the aroma compound production after completion of co-inoculated MLF. Values represent averages of triplicate treatments, analysed with GC-FID.

Volatile fatty acids

Acetic acid was quantitatively the predominant volatile fatty acid observed (**Table 3.15**). This was also found in the Merlot 2011 vintage and a study done by Lerm (2010). NT 50 and WE 372 showed increased concentrations of acetic acid, whereas Exotics showed lower concentrations of acetic acid, compared to the un-inoculated MLF control. The latter result was also found for the 2011 Merlot. MLF showed no impact on the acetic acid production for yeast NT 202. Malolactic fermentation showed decreased concentrations of propionic acid after completion of MLF for all treatments, compared to the un-inoculated MLF control (**Figure 3.27**). Exotics showed the lowest concentrations of propionic acid, regardless of the starter culture used. For butyric acid, MLF showed lower concentrations of these compounds for all treatments (**Figure 3.27**). No discernible differences were observed between treatments for decanoic acid.

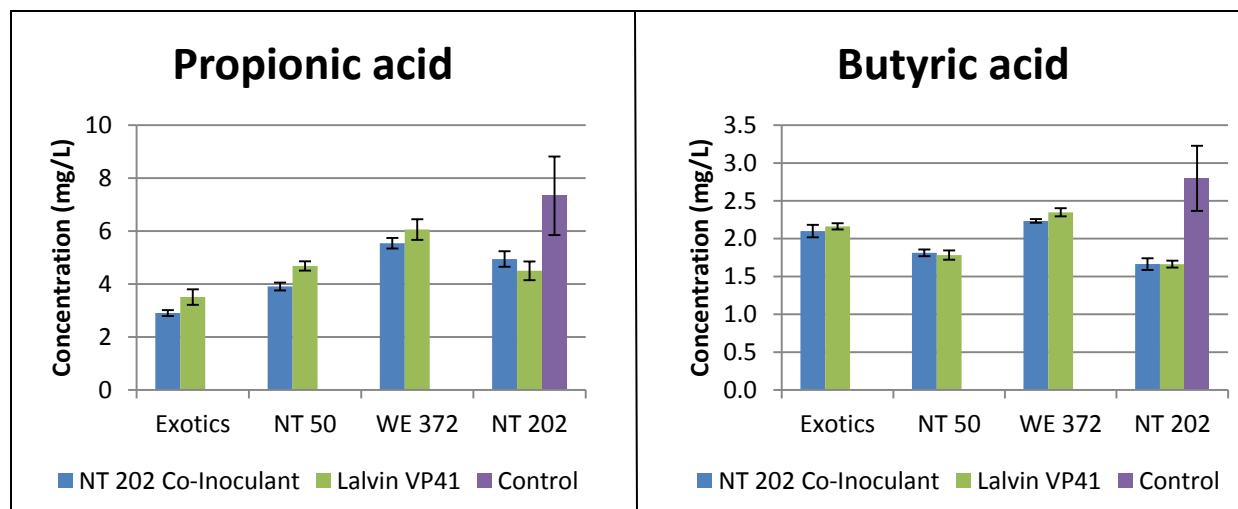


Figure 3.27 Volatile fatty acid production (mg/L) measured after completion of co-inoculated MLF for determining the impact of the yeast-bacterial combinations on the aroma compound production in the 2012 Shiraz. Values represent triplicate treatments, analysed with GC-FID.

Malolactic fermentation did not result in an increase in total volatile fatty acid for yeast treatment NT 202 (**Figure 3.28**). NT 50 showed the highest concentrations of total volatile fatty acid production, followed by WE 372, NT 202 and Exotics of which the latter treatment was lower than the un-inoculated MLF control. As previously mentioned, lower production of these fatty acids with low odour thresholds that can cause undesirable, pungent aromas at higher concentrations are favoured. At low concentrations these fatty acids may contribute to wine complexity (Malherbe, 2010).

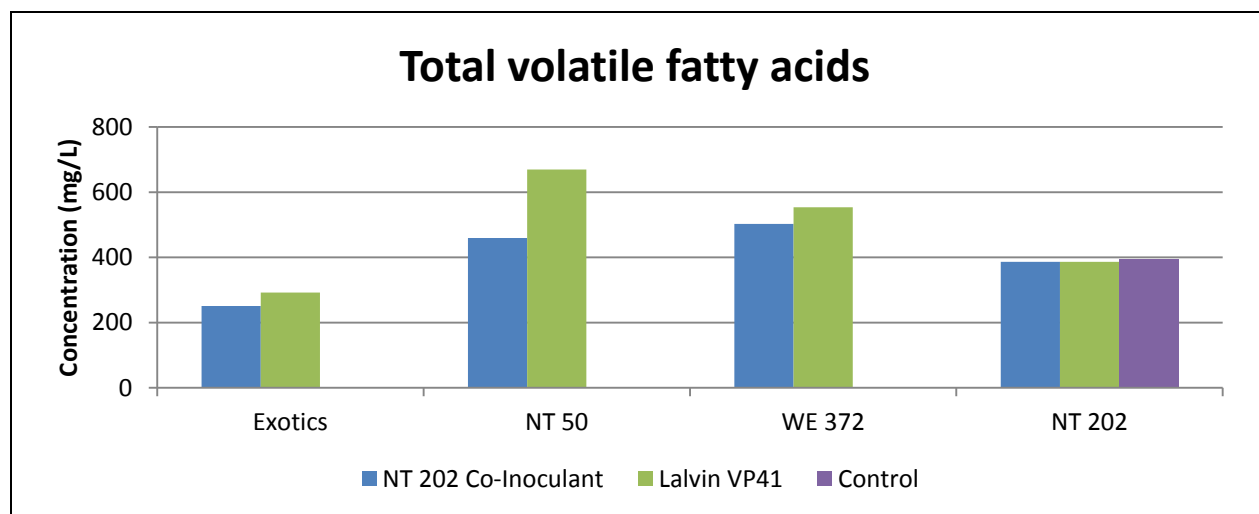


Figure 3.28 Total volatile fatty acids (mg/L) observed in the 2012 Shiraz for determining the impact of the yeast-bacterial combinations on the aroma compound production after completion of co-inoculated MLF. Values represent averages of triplicate treatments, analysed with GC-FID.

Monoterpenes

Of the 15 monoterpenes that were analysed in the 2012 Shiraz for determining the impact of the yeast-bacterial combinations on the aroma compound production with GC-FID, only linalool, citronellol, nerol, geraniol and β -farnesol 3 were detected (**Table 3.16**). The difference in the monoterpenes that were detected for this experiment compared to the 2011 Merlot indicates that cultivar plays a role in monoterpene production due to different precursor concentrations in different cultivars. This statement is supported by Michlmayr *et al.* (2012) who found that the precursors of important monoterpenes are synthesized during early berry development.

Table 3.16 Monoterpenes ($\mu\text{g/L}$) measured for determining the impact of the yeast-bacterial combinations on the aroma compound production in 2012 in Shiraz after completion of co-inoculated MLF using NT 202 Co-Inoculant and Lalvin VP41. Concentrations represent the average of triplicate treatments (standard deviations not shown), each analysed by GC-FID (nd: not detected).

	NT 202 Co-Inoculant				Lalvin VP41				Control
	Exotics	NT 50	WE 372	NT 202	Exotics	NT 50	WE 372	NT 202	NT 202
(\pm) Linalool	nd	nd	nd	20.87	nd	nd	24.46	22.82	23.17
Citronellol	9.88	12.01	11.21	12.72	10.71	12.71	11.44	13.62	13.76
Nerol	3.35	4.59	3.69	3.96	3.77	4.64	3.90	4.44	4.11
Geraniol	441.32	366.28	487.86	348.01	466.16	383.70	486.67	316.57	351.23
β -Farnesol 3	16.00	25.41	26.65	23.49	21.07	27.54	25.02	27.81	18.07
Total Monoterpenes	470.55	408.29	529.40	409.05	501.72	428.59	551.50	385.27	410.35

Linalool (rose) showed lower concentrations for treatments that underwent MLF compared to the un-inoculated control. This is in contrast to results obtained in a study by Knoll *et al.* (2012) that showed increased levels of linalool after MLF. Linalool was only detected for yeast treatments NT 202, irrespective of MLF treatment, and WE 372, when co-inoculated with Lalvin VP41. This might be attributed to difference in yeast strain metabolisms and/or yeast-bacterial interaction. Citronellol and nerol exhibited similar trends in production, of which the highest concentrations were observed for NT 50, followed by WE 372 and Exotics (**Figure 3.29**). The production of nerol was more dependent on yeast strain than LAB strain. Higher concentrations of nerol were observed with Lalvin VP41, regardless of yeast treatment, compared to the NT 202 Co-Inoculant. An overall trend of decreased citronellol (citrus) concentrations was observed for treatments that underwent MLF. Yeast treatments produced similar concentrations of citronellol, irrespective of the MLF starter culture used, in decreasing order: NT 202, NT 50, WE 372 and Exotics. The highest production of geraniol (rose, geranium) was observed for WE 372, followed by Exotics, NT 50 and NT 202, regardless of MLF starter culture used (**Figure 3.29**). This trend was also observed in the 2011 Merlot. WE 372 and Exotics, regardless of MLF

starter culture used, were the only two treatments to produce higher concentrations of geraniol than the un-inoculated MLF control. The high levels of geraniol production exhibited by WE 372 and Exotics were also observed in the 2011 Merlot. Malolactic fermentation led to an overall increase in β -Farnesol 3 concentrations compared to the un-inoculated MLF control of which Exotics exhibited lower concentrations than the remaining yeast treatments (**Figure 3.29**).

There was an overall trend for co-inoculation with Lalvin VP41 to produce more of a particular monoterpene, irrespective of the yeast treatment, compared to NT 202 Co-Inoculant indicating that the presence of *L. plantarum* in the NT 202 Co-Inoculant had an influence on monoterpene production. This might be attributed to possible yeast-bacterial interactions between the yeasts and the *L. plantarum* present in the NT 202 Co-Inoculant. In general the production of monoterpenes was more yeast strain than LAB strain dependent. WE 372 showed the highest concentrations of total monoterpenes produced, regardless of MLF starter culture used, followed by Exotics (**Figure 3.29**).

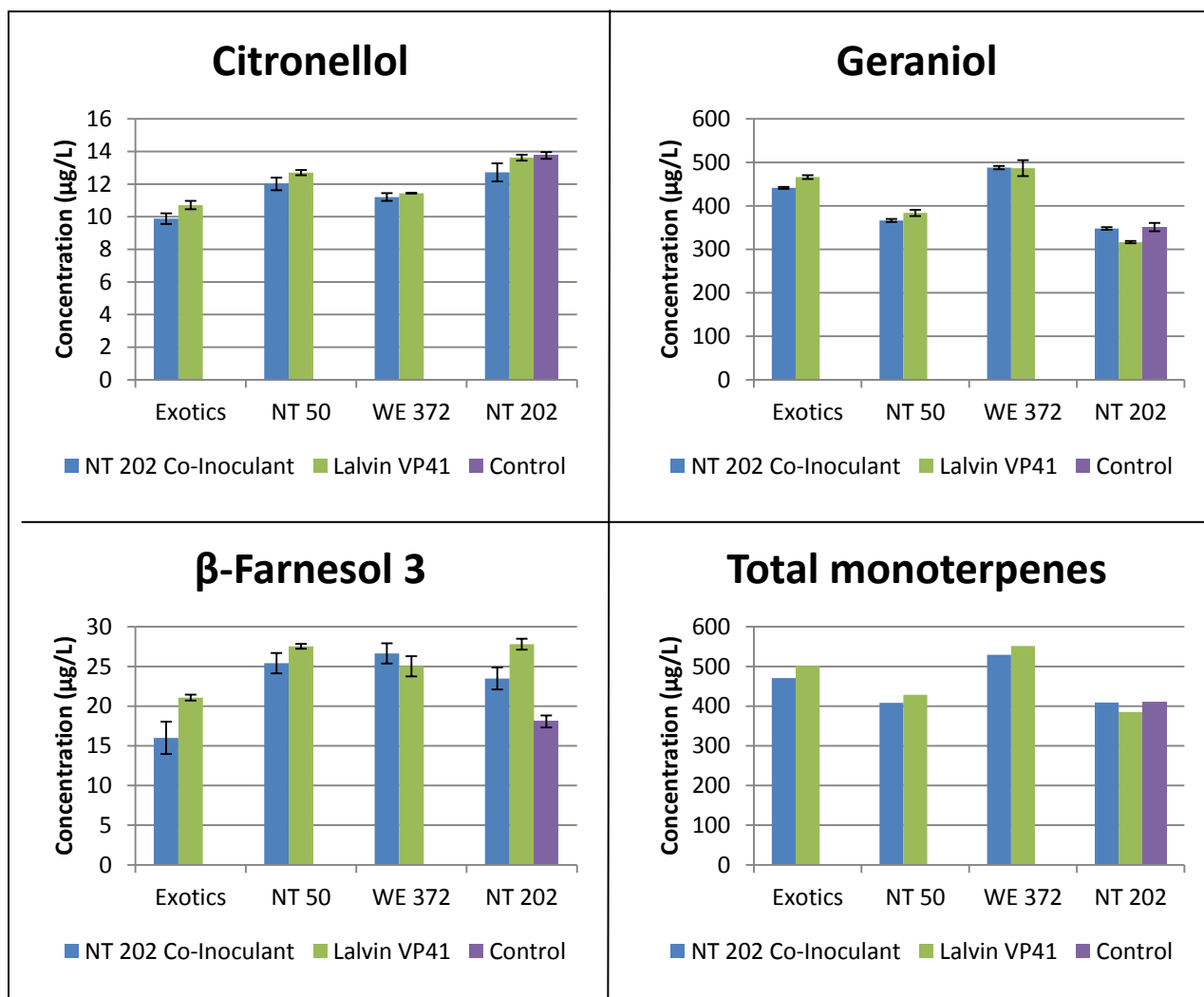


Figure 3.29 Monoterpene production ($\mu\text{g/L}$) measured in the 2012 Shiraz for determining the impact of the yeast-bacterial combinations on the aroma compound production after completion of co-inoculated MLF. Values represent triplicate treatments, analysed by GC-FID.

Carbonyl Compounds

Results of the GC-MS-detected carbonyl compounds, using GC-MS, for determining the impact of the yeast-bacterial combinations on the aroma compound production in the 2012 Shiraz wines are listed in **Table 3.17** and **Figure 3.30**.

Table 3.17 Carbonyl compounds (mg/L) measured for determining the impact of the yeast-bacterial combinations on the aroma compound production in 2012 in Shiraz after completion of co-inoculated MLF. Values represent triplicate treatments (standard deviations not shown), analysed by GC-MS (nd: not detected).

	NT 202 Co-Inoculant				Lalvin VP41				Control
	Exotics	NT 50	WE 372	NT 202	Exotics	NT 50	WE 372	NT 202	NT 202
Diacetyl	5.19	11.78	3.87	4.43	3.32	11.10	4.57	5.16	1.28
2,3-Pentadione	16.35	9.56	11.62	9.64	14.65	9.94	11.23	9.46	12.81
Acetoin	28.36	36.29	23.37	28.09	19.03	48.64	15.92	19.07	29.90
Total Carbonyl Compounds	49.90	57.63	38.86	42.16	37.00	69.68	31.72	33.69	43.99

As observed in the 2011 Merlot and by Lerm (2010), MLF in the 2012 Shiraz for determining the impact of the yeast-bacterial combinations on the aroma compound production resulted in increased in diacetyl (buttery, nutty, butterscotch) concentrations (**Figure 3.30**). The highest diacetyl concentration (compared to the un-inoculated MLF control) was observed for yeast treatment NT 50, which was also the case observed in the 2011 Merlot. In terms of diacetyl, the remaining treatments, except the un-inoculated MLF control, showed similar levels of production. The highest concentration of 2,3-pentadione was observed for Exotics, regardless of MLF starter culture used, which was also the only treatment to produce higher concentrations than the un-inoculated MLF control. Similar trends in 2,3-pentadione production were observed for the yeasts, regardless of MLF starter culture used. The highest production of acetoin (butter, cream) was observed for NT 50 in combination with Lalvin VP41. Treatments with NT 50, irrespective of the MLF starter culture, were the only treatments that showed higher acetoin concentrations, compared to the un-inoculated MLF control. Yeast treatment NT 50, co-inoculated with NT 202 Co-Inoculant, also proved to produce high levels of diacetyl and acetoin compared to the remaining treatments in the 2011 Merlot. As expected and previously discussed, the levels of acetoin were observed to be higher than the diacetyl levels for all treatments.

Trends observed for total carbonyl compounds will mimic that of acetoin due to its quantitative predominance (**Figure 3.30**). Yeast treatment NT 50 was the highest producer of total carbonyl compounds. This was also observed in the 2011 Merlot results. NT 202 Co-Inoculant showed higher levels of total carbonyl compounds (compared to NT 202 Co-Inoculant) for all yeasts, except NT 50, where co-inoculation with Lalvin VP41 showed higher levels of production.

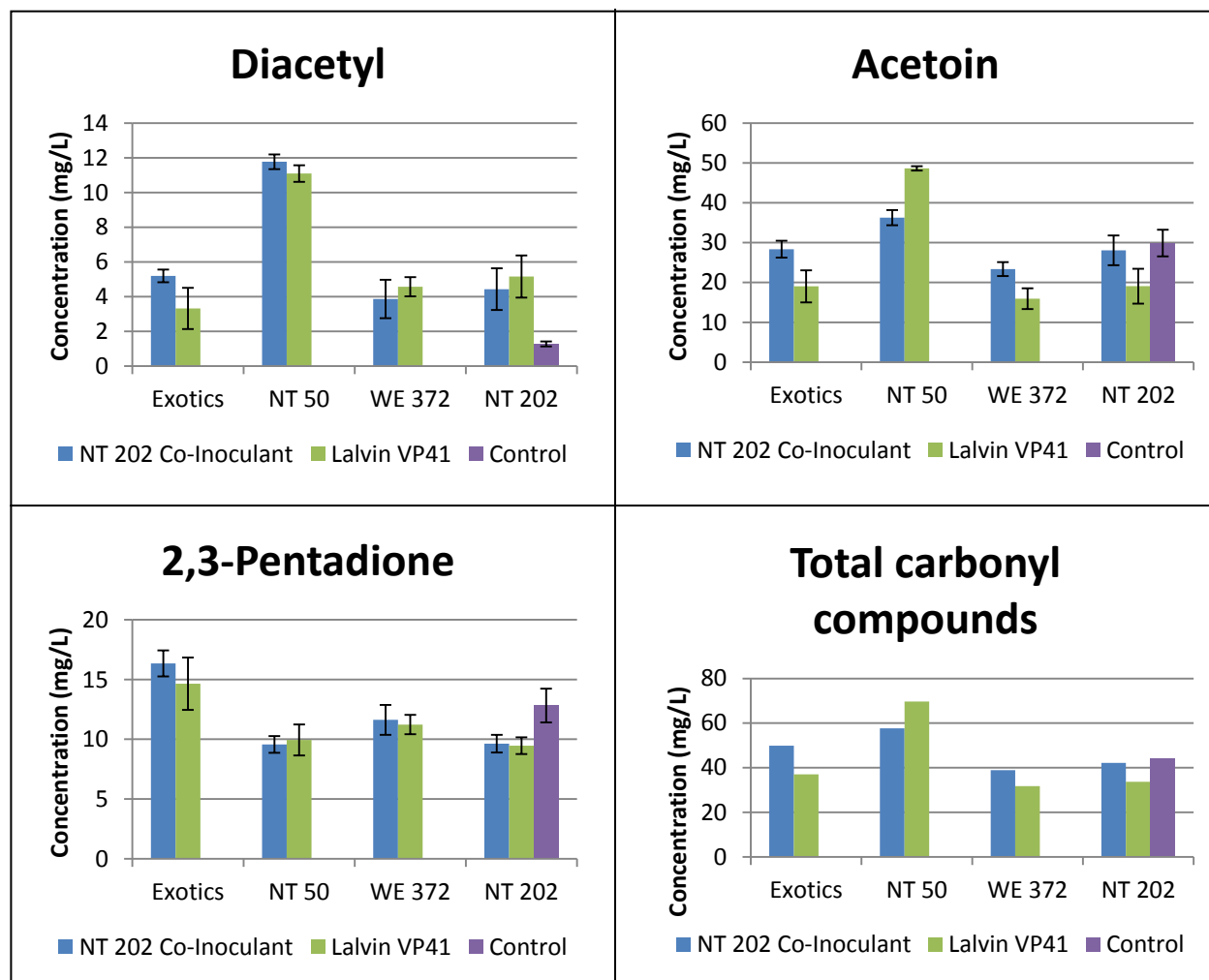


Figure 3.30 Carbonyl compound production (mg/L) of the different yeasts and bacteria combinations analysed for determining the impact of the yeast-bacterial combinations on the aroma compound production in the 2012 Shiraz after completion of MLF. Values represent triplicate treatments, analysed by GC-MS.

Principal component analysis was conducted on the GC-FID and GC-MS generated data of the 2012 Shiraz wines to determine the impact of the yeast-bacterial combinations on the aroma compound production to investigate correlations between different treatment samples and aroma attributes during co-inoculation with NT 202 Co-Inoculant (**Figure 3.31**).

A clear negative correlation is observed for the un-inoculated MLF samples and MLF-associated descriptors such as diacetyl, acetoin and ethyl lactate across PC2. This observation supports findings already discussed for the 2012 Shiraz for determining the impact of the yeast-bacterial combinations on aroma compound production.

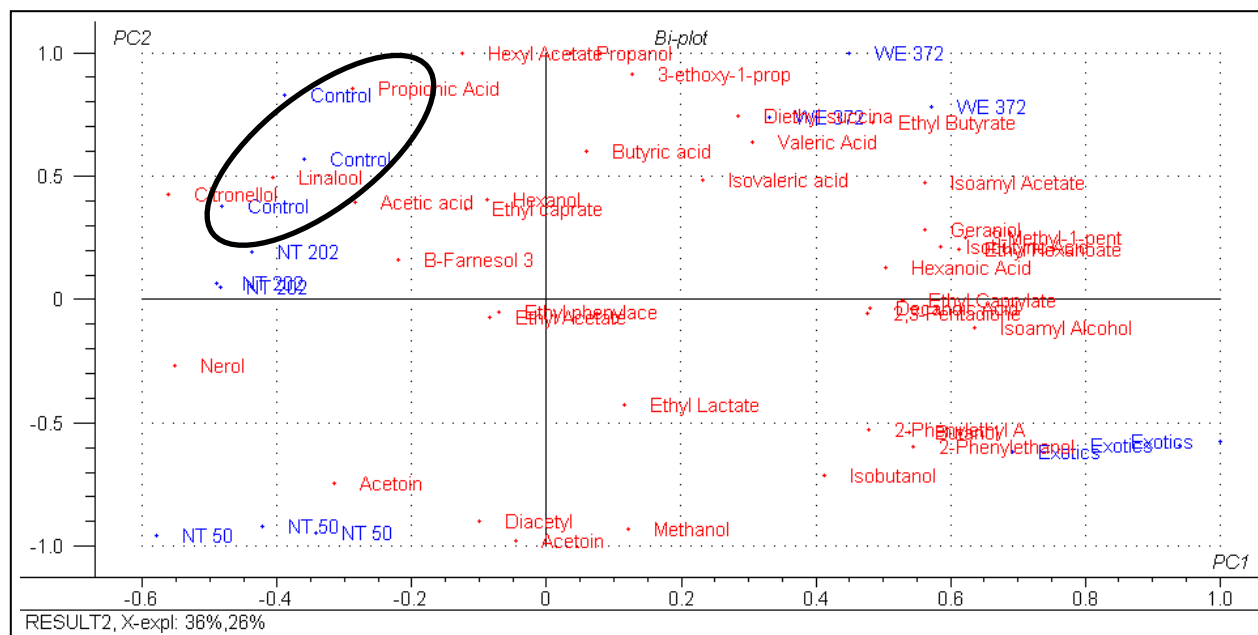


Figure 3.31 PCA Bi-plot of scores (in blue) and loadings (in red) of the GC-FID and GC-MS generated data for the 2012 Shiraz for determining the impact of the yeast-bacterial combinations on the aroma compound production after completion of co-inoculated MLF using NT 202 Co-Inoculant. NT 202 yeast was used for the un-inoculated MLF control.

Impact of additives on aroma compound production

Results for the major volatile (esters, higher alcohols and volatile fatty acids) analyses done on the 2012 Shiraz for determining the impact of additives on the aroma compound production, using GC-FID, are listed in **Table 3.18**. No discernible differences were observed for ethyl acetate, ethyl butyrate, isoamyl alcohol or decanoic acid and will therefore not be discussed. Due to a lack of studies concerning the impact of oenological additives on wine aroma compounds when used during co-inoculated MLF, correlations of results of this study with others are limited.

Table 3.18 Major volatile (Esters, higher alcohols and volatile fatty acids) production (mg/L) measured after completion of co-inoculated MLF in the 2012 Shiraz using NT 202 yeast with the NT 202 Co-Inoculant for determining the impact of additives on the aroma compound production. Values represent the average of triplicate treatments (standard deviations not shown), analysed by GC-FID. (nd: not detected)

	Extraferm	Natuferm	Claristar	OptiMalo Plus	Bactiv-aid	Control (DAP)	Nutrivin
Esters							
Ethyl Acetate	144.01	131.17	133.93	141.62	146.06	136.85	140.42
Ethyl Butyrate	0.47	0.46	0.49	0.49	0.47	0.49	0.47
Isoamyl Acetate	4.84	4.47	5.00	4.73	4.72	5.34	5.04
Ethyl Hexanoate	0.54	0.53	0.58	0.57	0.55	0.57	0.49
Ethyl Lactate	35.33	34.77	32.35	36.23	34.62	40.00	41.56
Ethyl Caprylate	0.18	0.18	0.22	0.20	0.19	0.20	0.17
Ethyl caprate	nd	0.05	nd	nd	0.05	0.05	nd
Diethyl succinate	0.61	0.63	0.57	0.59	0.56	0.59	0.58
2-Phenylethyl Acetate	0.58	0.57	0.59	0.56	0.57	0.60	0.54
Total Esters	186.55	172.82	173.72	184.99	187.80	184.68	189.27
Higher Higher Alcohols							
Methanol	229.27	214.14	219.30	240.15	230.24	203.42	208.69
Propanol	133.53	141.83	123.22	136.46	132.95	156.52	196.17
Isobutanol	33.08	34.49	33.28	33.65	32.85	34.67	38.74
Butanol	4.36	4.19	4.24	4.17	4.37	4.16	4.63
Isoamyl Alcohol	338.06	333.18	335.21	338.12	334.03	337.46	339.68
4-Methyl-1-pentanol	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3-Methyl-1-pentanol	0.21	0.19	0.22	0.23	0.22	0.22	0.19
Hexanol	3.20	3.42	3.19	3.52	3.19	3.39	3.01
3-ethoxy-1-propanol	3.96	4.60	3.75	4.27	4.38	5.21	6.07
2-Phenylethanol	59.86	59.21	59.46	57.68	57.51	55.49	51.16
Total Higher Alcohols	805.54	795.24	781.87	818.25	799.75	800.55	848.35
Volatile Fatty Acids							
Acetic acid	326.64	378.97	280.20	323.82	335.95	287.62	372.70
Propionic Acid	7.87	4.13	3.39	3.51	3.73	4.10	4.63
Isobutyric Acid	1.61	1.85	1.61	1.54	1.58	1.70	1.92
Butyric acid	1.57	1.66	1.52	1.60	1.57	1.68	1.66
Isovaleric acid	2.54	2.84	2.10	2.28	2.41	2.49	2.21
Valeric Acid	0.43	0.44	0.43	0.44	0.46	0.47	0.53
Hexanoic Acid	2.29	2.30	2.32	2.35	2.25	2.40	2.03
Decanoic Acid	0.58	0.59	0.60	0.60	0.58	0.63	0.59
Total Volatile Fatty Acids	343.53	392.78	292.19	336.14	348.54	301.09	386.27

Esters

Little variation in produced quantities was observed for most of the detected esters (**Table 3.18**), except ethyl lactate and 2-phenylethyl acetate (**Figure 3.32**). The yeast nutrient, Nutrivin showed the highest concentration of ethyl lactate whereas the clarifying agent, Claristar, showed the lowest concentration. This indicates that standard DAP or Nutrivin additions favours the binding of lactate and ethanol in the wine during co-inoculated MLF more than the remaining additions and addition of the former treatments will influence the mouthfeel of the wine more than the remaining treatments. The lowest concentration of 2-phenylethyl acetate was observed for Nutrivin, which showed a lower concentration than that observed for the remaining

treatments, except for OptiMalo Plus. The highest concentrations of 2-phenylethyl acetate were observed for Claristar and the Control (DAP).

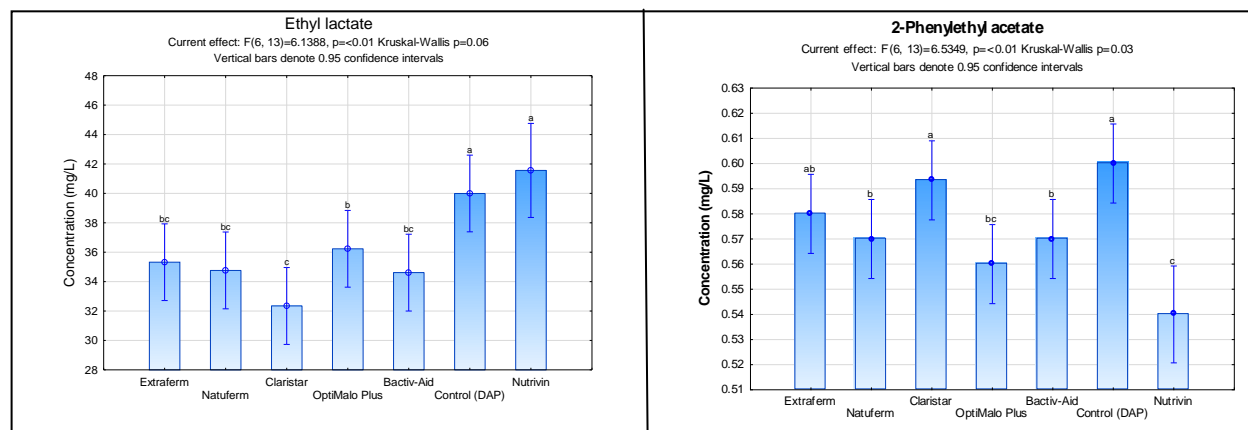


Figure 3.32 Esters (mg/L) measured after completion of co-inoculated MLF for determining the impact of additives on the aroma compound production in the 2012 Shiraz. Values represent the average of triplicate treatments measured using GC-FID. Vertical bars denote 0.95 confidence intervals.

The highest total ester concentration measured after completion of co-inoculated MLF was observed for Nutrivin, followed by Bactiv-Aid, Extraferm, OptiMalo Plus, Control (DAP), Claristar and Natuferm (**Figure 3.33**). Compared to the control DAP addition, Natuferm (100% inactivated yeast) and Claristar (mannoproteins) are the only two treatments that resulted in lower total esters.

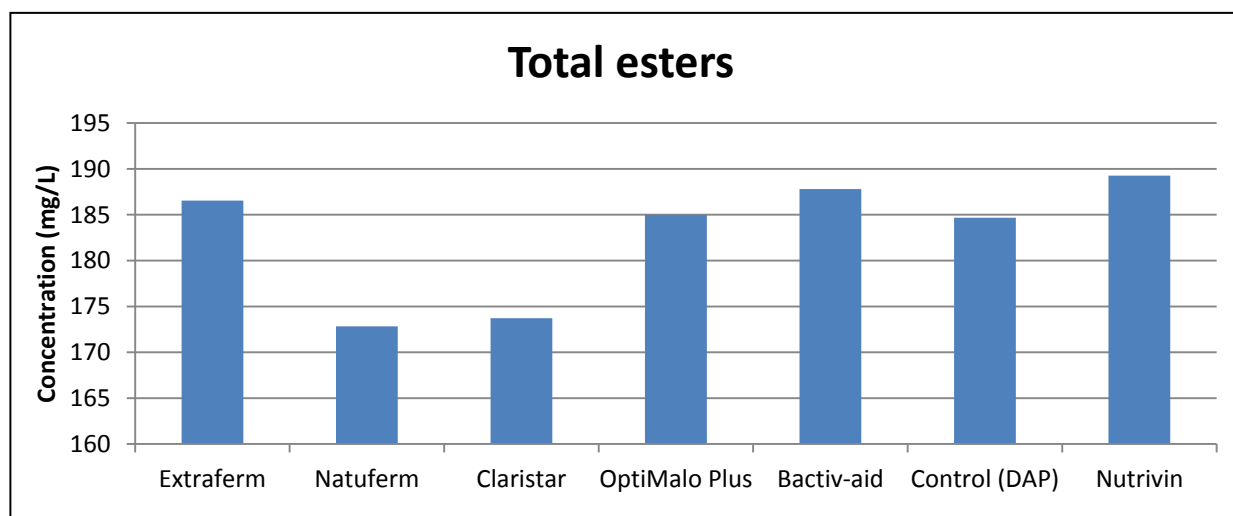


Figure 3.33 Total ester production (mg/L) measured after completion of co-inoculated MLF, using NT 202 with NT 202 Co-Inoculant, for determining the impact of additives on the aroma compound production in the 2012 Shiraz. Values represent the average of triplicate treatments measured using GC-FID.

Higher alcohols

As with the two previous experiments of this study and other studies already mentioned, isoamyl alcohol was quantitatively predominant. However, little variation between treatments was observed for all detected higher alcohols, except propanol, isobutanol, butanol and 3-ethoxy-1-propanol that showed similar trends in production (**Table 3.18**). E.g. for 3-ethoxy-1-propanol, that gives a fruity character to the wine (Malherbe, 2010), the highest concentration was observed for the yeast nutrients Nutrivin, followed by the Control (DAP) (**Figure 3.34**). The remaining treatments showed little variation between one another.

The highest total higher alcohol production was observed for Nutrivin, followed by OptiMalo Plus, Extraferm, Control (DAP), Bactiv-Aid, Natuferm and Claristar (**Figure 3.35**).

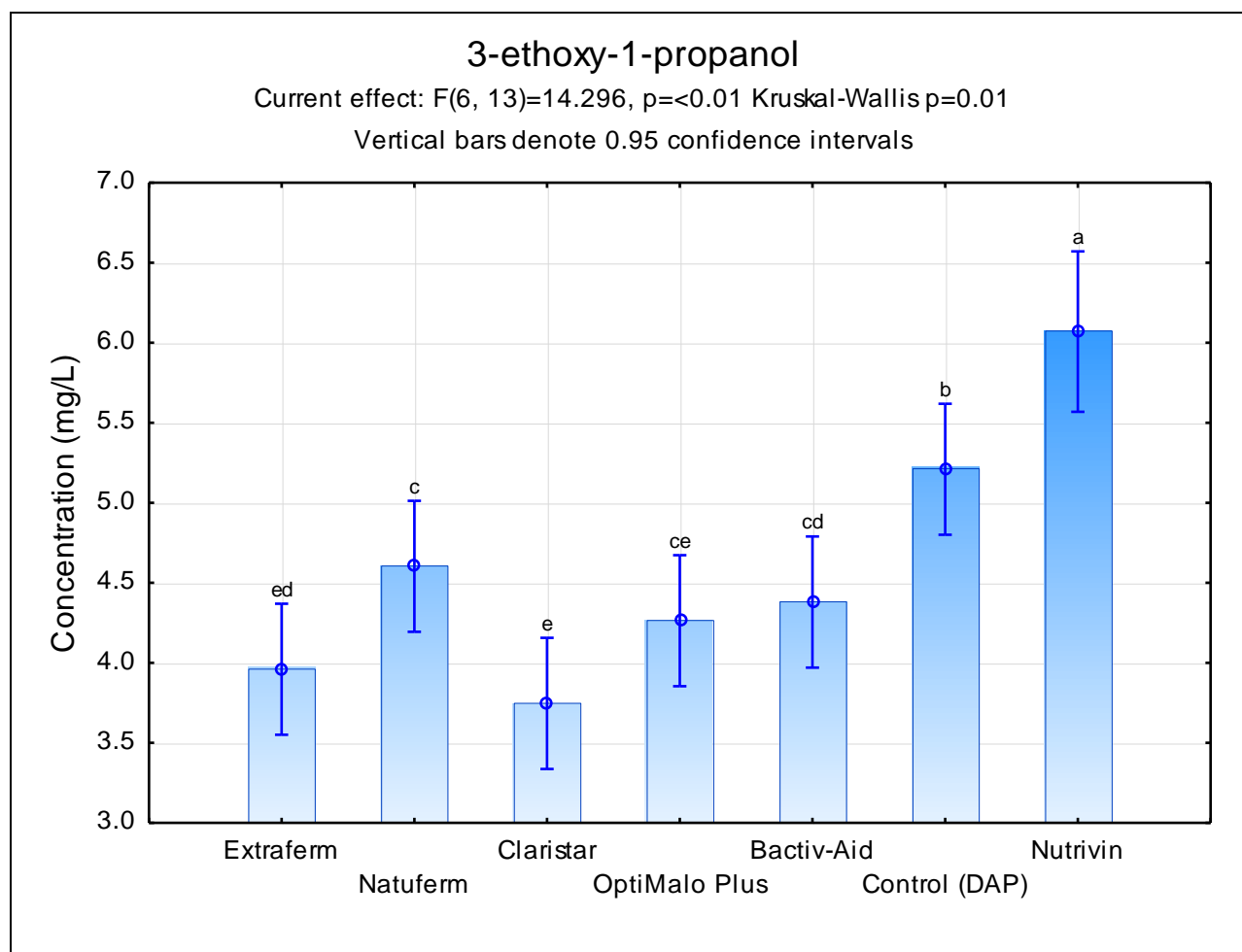


Figure 3.34 Higher alcohols (mg/L) measured after completion of co-inoculated MLF for determining the impact of additives on the aroma compound production in the 2012 Shiraz using NT 202 with NT 202 Co-Inoculant. Values represent the average of triplicate treatments measured using GC-FID. Vertical bars denote 0.95 confidence intervals.

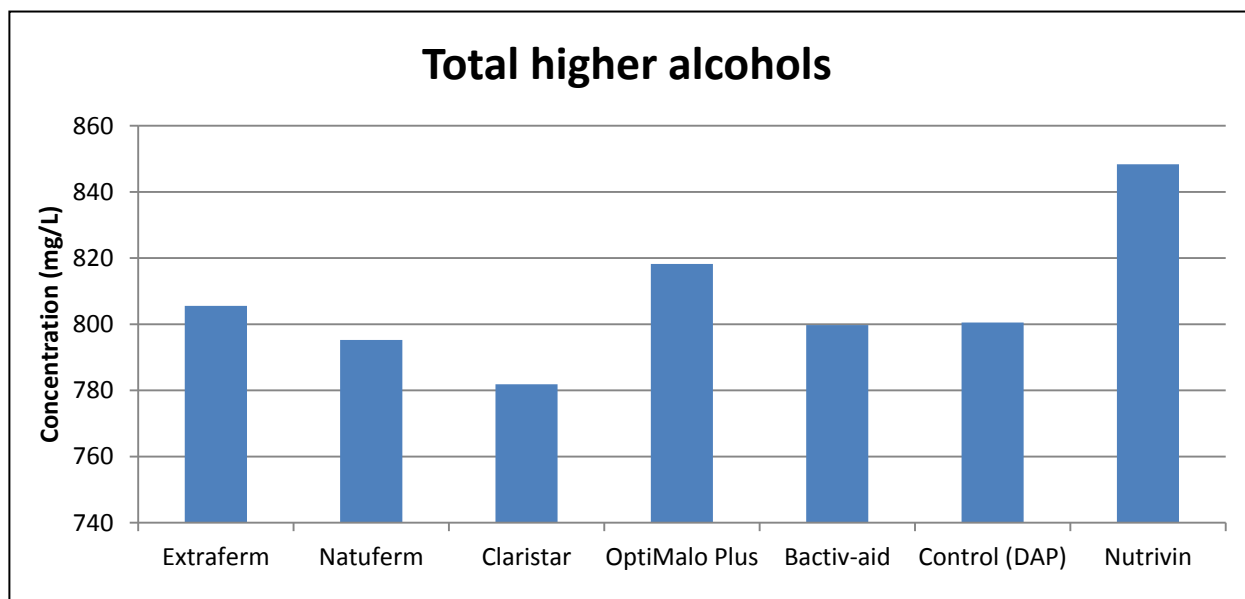


Figure 3.35 Total higher alcohol production (mg/L) measured after completion of co-inoculated MLF, using NT 202 with NT 202 Co-Inoculant, for determining the impact of additives on the aroma compound production in the 2012 Shiraz. Values represent the average of triplicate treatments measured using GC-FID.

Volatile fatty acids

As with the previously discussed results, acetic acid was quantitatively the predominant acid observed. Natuferm showed the highest concentration of acetic acid, after completion of co-inoculated MLF, similar to Nutrivin, followed by Extraferm and Bactiv-Aid, OptiMalo Plus and control (DAP) and Claristar (**Figure 3.36**). Acetic acid quantitatively predominated among the volatile fatty acids. The highest concentration of propionic acid was observed for Nutrivin, whereas little variation was observed for the remaining treatments (**Table 3.18**). Little difference in concentrations between treatments was observed for isobutyric, butyric and isovaleric acids. The highest concentration of valeric acid was observed for Nutrivin compared to the remaining treatments, which showed no discernible differences in concentrations. The lowest concentration of hexanoic acid was observed for Nutrivin compared to the remaining treatments that showed little difference in concentrations.

The highest total volatile fatty acid production was observed for Natuferm, followed by Nutrivin, Bactiv-Aid, Extraferm, OptiMalo Plus, Control (DAP) and Claristar (**Figure 3.37**). The little variation in concentration of volatile fatty acid production may be attributed to the use of co-inoculation and the sufficient availability of nutrients available at onset of and throughout AF as well as MLF.

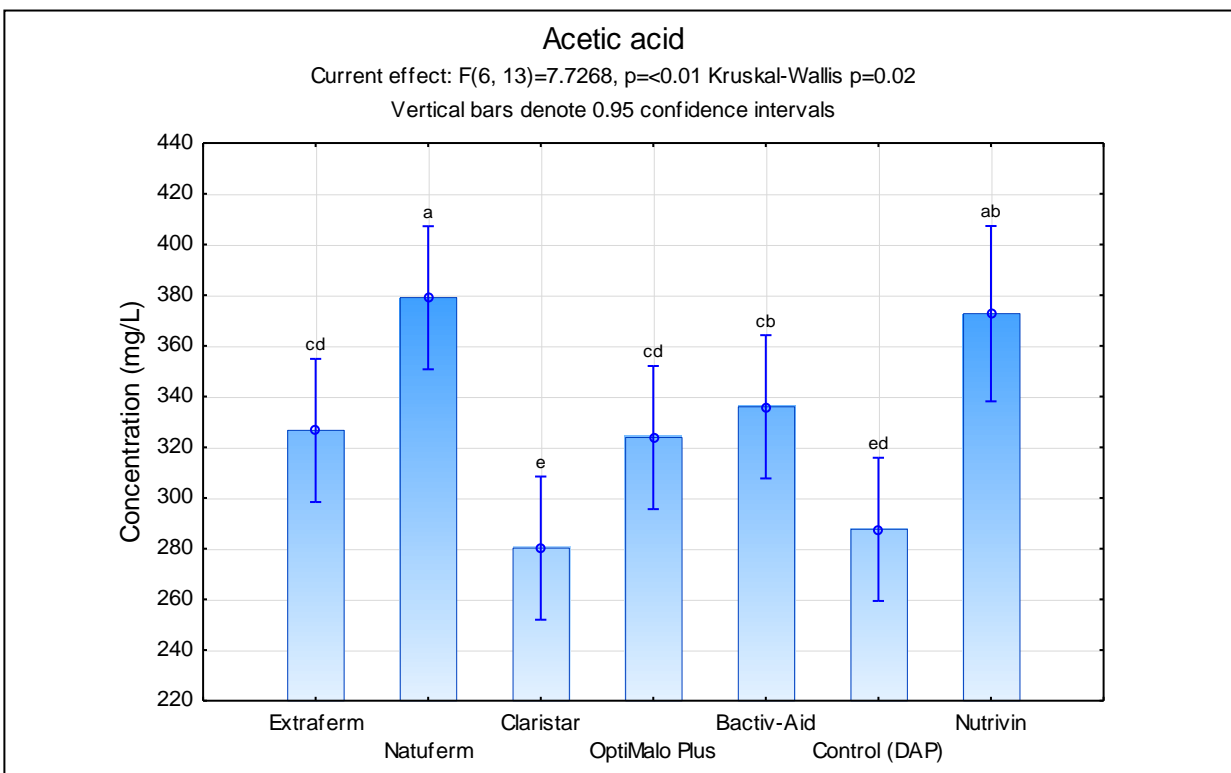


Figure 3.36 Acetic acid (mg/L) measured after completion of co-inoculated MLF for determining the impact of additives on the aroma compound production in the 2012 Shiraz using NT 202 with NT 202 Co-Inoculant. Values represent the average of triplicate treatments measured using GC-FID. Vertical bars denote 0.95 confidence intervals.

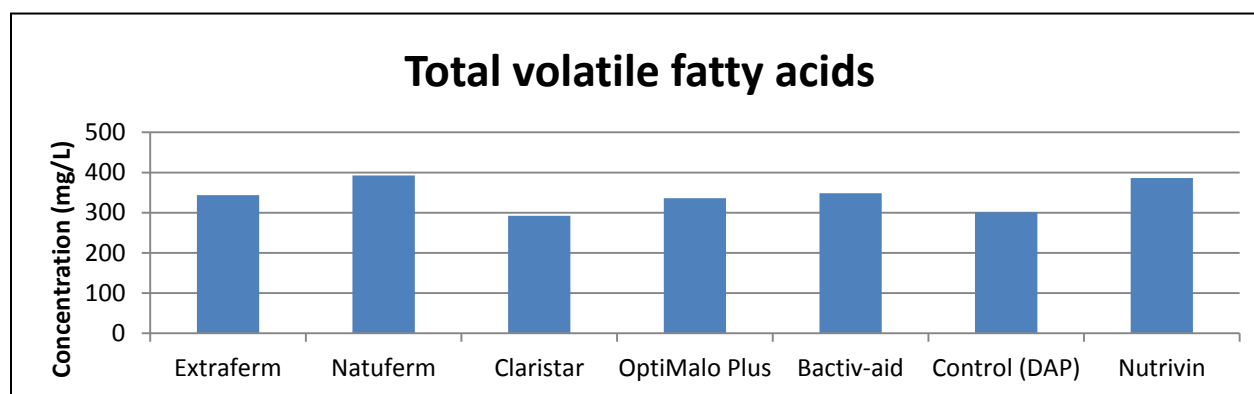


Figure 3.37 Total volatile fatty acid production (mg/L) measured after completion of co-inoculated MLF, using NT 202 with NT 202 Co-Inoculant, for determining the impact of additives on the aroma compound production in the 2012 Shiraz. Values represent the average of triplicate treatments measured using GC-FID.

Monoterpenes

Of the 15 monoterpenes that were analysed during the 2012 vintage in Shiraz for determining the impact of additives on the aroma compound production, only linalool, citronellol, nerol, geraniol and β -farnesol 3 were detected (**Table 3.19** and **Figure 3.38**). It was also only these monoterpenes that were detected for the other part of this study conducted in the 2012 Shiraz that tested the impact of yeast-bacterial combinations on the aroma compound production during co-inoculation. Again this support the previously mentioned statement that cultivar influences monoterpene production.

Table 3.19 Monoterpenes ($\mu\text{g/L}$) measured for determining the impact of additives on the aroma compound production in 2012 in Shiraz after completion of co-inoculated MLF using NT 202 with NT 202 Co-Inoculant. Values represent the average of triplicate treatments (standard deviations not shown), each analysed by GC-FID. (nd: not detected)

	Extraferm	Natuferm	Claristar	OptiMalo Plus	Bactiv-Aid	Control (DAP)	Nutrivin
\pm Linalool	9.46	16.59	16.43	13.39	16.14	15.13	20.87
Citronellol	13.24	14.47	13.77	13.62	14.29	13.30	12.72
Nerol	4.47	4.52	4.23	4.25	4.46	4.21	3.96
Geraniol	290.67	325.61	332.83	347.26	326.56	374.89	348.01
β -Farnesol 3	26.20	26.80	24.97	24.78	26.72	28.06	23.49
Total Monoterpenes	344.04	388.00	392.23	403.31	388.17	435.59	409.05

The highest concentration of linalool (rose) was observed for the yeast nutrient, Nutrivin, whereas the lowest concentration was observed for the detoxifying agent, Extraferm (**Figure 3.38**). Similar levels of linalool were observed for the remaining treatments. In terms of Citronellol (citrus) production, the yeast nutrient Natuferm showed the highest concentration which was similar to concentrations observed for the clarifying agent Claristar, and the bacterial nutrients OptiMalo Plus and Bactiv-Aid (**Figure 3.38**). The lowest concentration of citronellol was observed for Nutrivin of which the concentrations were similar to that observed for Extraferm and the control, where DAP were added as yeast nutrient. Similar trends in production were observed for nerol and β -Farnesol 3 where the highest concentrations were observed for Extraferm, Natuferm and Bactiv-Aid and the lowest concentrations observed for Nutrivin (**Figure 3.38**). In terms of geraniol (rose, geranium) production, the highest concentration was observed for the Control (DAP), the lowest for Extraferm, whereas the remaining treatments showed similar levels of production (**Figure 3.38**).

In general, higher concentrations of geraniol, compared to citronellol, were observed for all treatments. This could be due to β -glucosidase activity of the yeast to reduce geraniol to citronellol or chemical hydrolysis of the bound forms (Carrau *et al.*, 2005). Similar trends in

production were observed for the total monoterpenes due the quantitative predominance of geraniol (**Table 3.19**). In short, the highest concentration of total monoterpenes was observed for Control (DAP), followed by Nutrivin, OptiMalo Plus, Claristar, Bactiv-Aid, Natuferm and Extraferm. In general no specific trend was observed for the different types of additives.

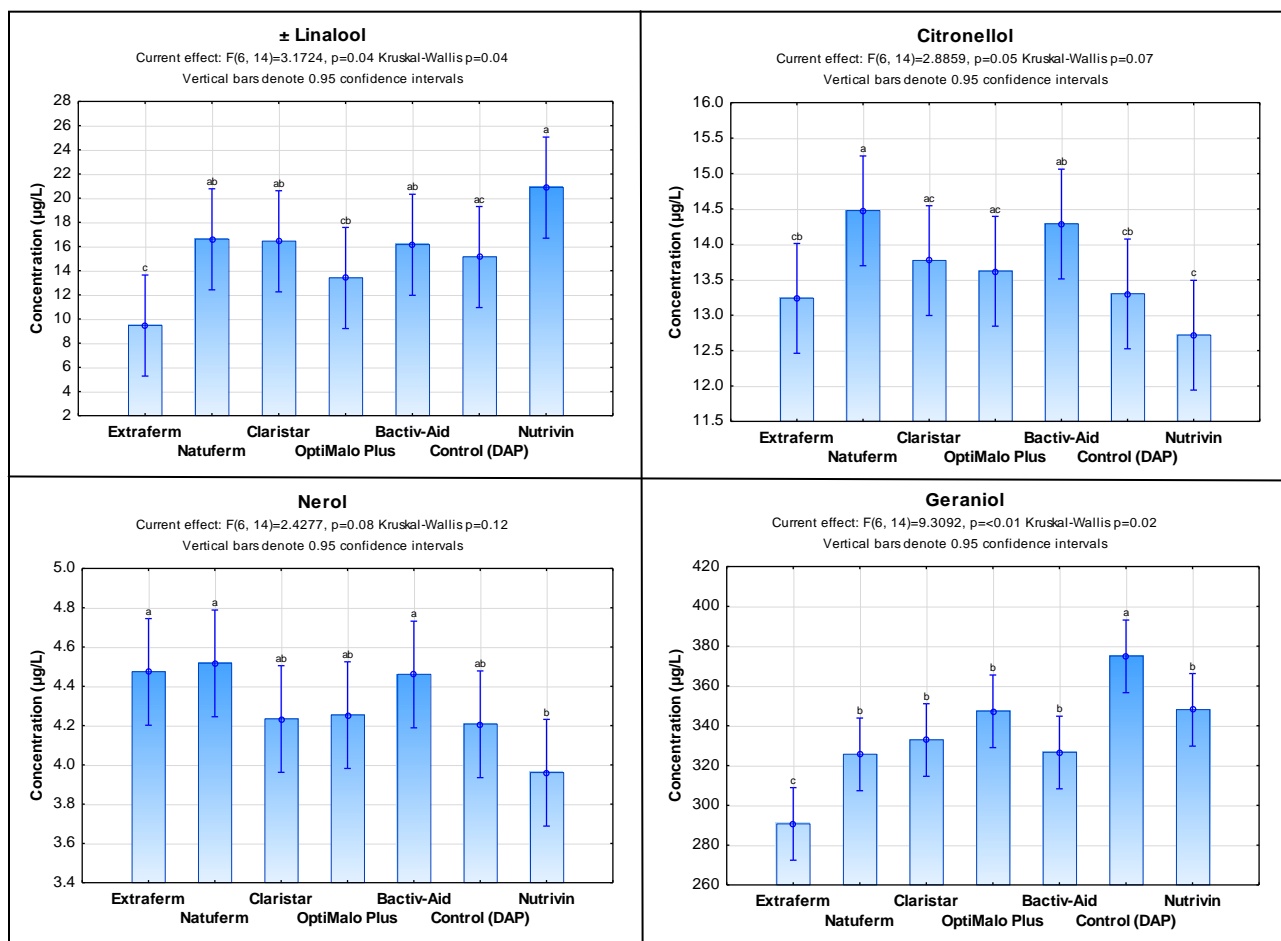


Figure 3.38 Monoterpene production (µg/L) measured in the 2012 Shiraz for determining the impact of additives on the aroma compound production after completion of co-inoculated MLF using NT 202 with NT 202 Co-Inoculant. Values represent triplicate treatments, analysed by GC-FID. Vertical bars denote 0.95 confidence intervals.

Carbonyl compounds

The principal carbonyl compounds that were analysed in the 2012 Shiraz for determining the impact of additives on the aroma compound production by GC-MS are listed in **Table 3.20** and **Figure 3.39**. Similar trends in production were observed for diacetyl (butter, nutty, butterscotch) and 2,3-pentadione (buttery, cream) for the different treatments, except for Control (DAP) and Nutrivin. Control (DAP) and Nutrivin treatments showed higher levels (compared to the remaining treatments) of 2,3-pentadione than for diacetyl. Interestingly enough, the highest and

lowest concentrations of diacetyl were observed for the bacterial nutrients Bactiv-Aid and OptiMalo Plus, respectively. This was also observed for 2,3-pentadione, except for Claristar, the Control (DAP) and Nutrivin, which showed similar high levels of production compared to Bactiv-Aid. The highest concentration of acetoin (butter, cream) was observed for Nutrivin and the lowest for Bactiv-Aid. This was in contrast to diacetyl production where Bactiv-Aid showed the highest concentration. Due to its quantitative predominance, trends for total concentrations of carbonyl compounds will mimic those of acetoin, thus Nutrivin and Claristar were the highest producers of total carbonyl compounds.

Table 3.20 Carbonyl compounds (mg/L) measured for determining the impact of additives on the aroma compound production in 2012 in Shiraz after completion of co-inoculated MLF using NT 202 with NT 202 Co-Inoculant. Values represent the average of triplicate treatments (standard deviations not shown), analysed by GC-MS. (nd: not detected)

	Extraferm	Natuferm	Claristar	OptiMalo	Bactiv-Aid	Control (DAP)	Nutrivin
Diacetyl	4.79	4.33	6.06	2.94	6.35	4.57	4.43
2,3-Pentadione	8.41	6.78	9.64	6.18	9.64	9.18	9.64
Acetoin	20.75	20.50	26.76	24.17	17.36	22.29	28.09
Total Carbonyl Compounds	33.96	31.62	42.46	33.29	33.36	36.04	42.16

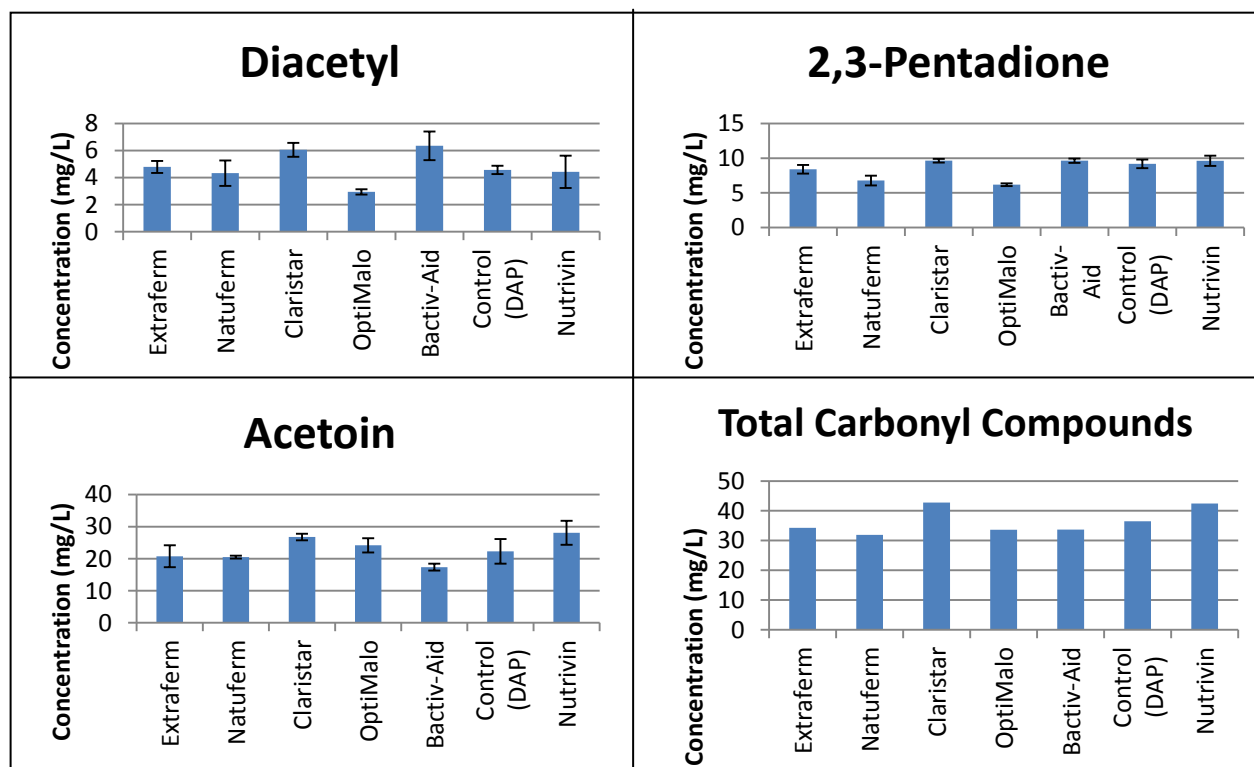


Figure 3.39 Carbonyl compound production (mg/L) measured in the 2012 Shiraz for determining the impact of additives on the aroma compound production after completion of co-inoculated MLF using NT 202 with NT 202 Co-Inoculant. Values represent triplicate treatments, analysed by GC-MS.

Principal component analysis was conducted on the GC-FID and GC-MS generated data of the 2012 Shiraz wines to investigate correlations between different additive treatment samples, co-inoculated using NT 202 with NT 202 Co-Inoculant, and aroma attributes (**Figure 3.40**).

As indicated by the black oval in **Figure 3.40**, Nutrivin did not seem to positively correlate very well to the aroma attributes. The Control (DAP) duplicate samples did not correlate very well with one another, thereby limiting specific observations in terms of correlations to specific aroma attributes.

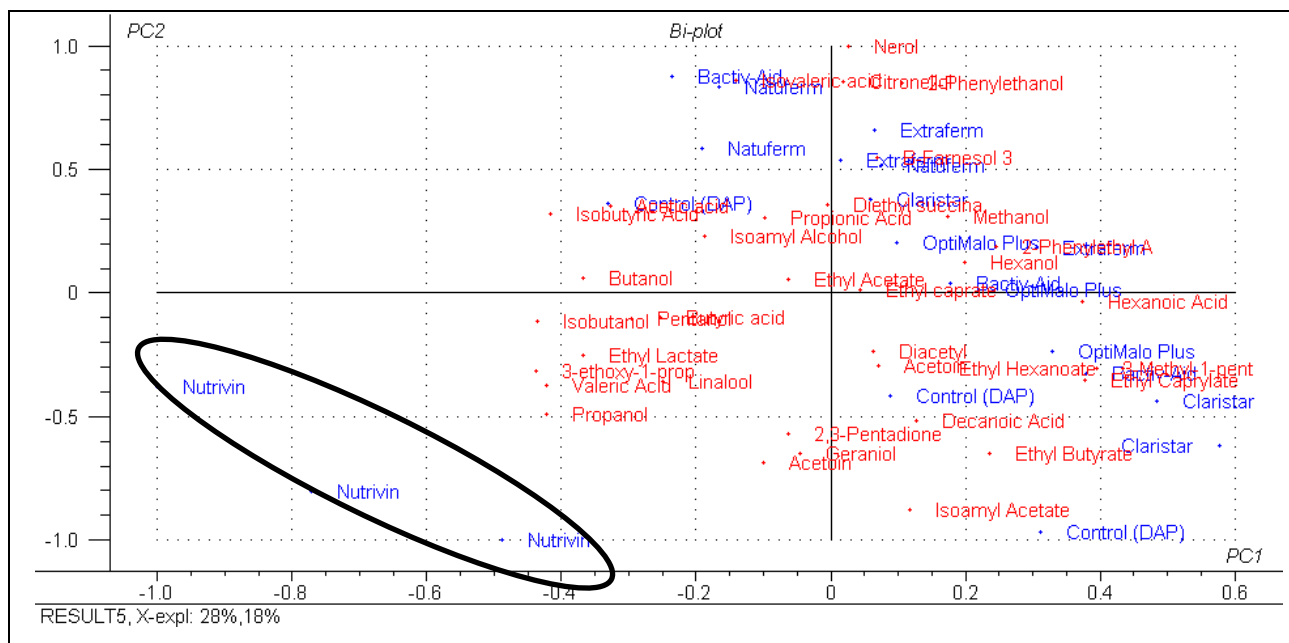


Figure 3.40 PCA Bi-plot of scores (in red) and loadings (in blue) of the GC-FID and GC-MS generated data for the 2012 Shiraz for determining the impact of additives on the aroma compound production after completion of co-inoculated MLF using NT 202 with NT 202 Co-Inoculant.

3.4 Conclusions

Inoculation of malolactic starter cultures is common practice in many wineries today. Mixed MLF starter cultures such as the NT 202 Co-Inoculant containing *L. plantarum* as well as *O. oeni*, marketed for conducting co-inoculated MLF showed positive compatibility with various other yeast strains e.g. WE 372 and Exotics in both Merlot and Shiraz. Re-evaluation in Chardonnay is also advised. Co-inoculated MLF showed positive aroma changes in the red wines. Further research is still needed to evaluate different inoculation times, include more red wine cultivars (commonly used in South Africa), in bigger volumes together with barrel aging. These potential evaluations would also benefit from more in-depth sensory analyses e.g. quantitative descriptive

analysis (QDA). Additions during co-inoculated MLF showed no negative or positive effect on MLF and associated aroma compounds.

To conclude, this study found that co-inoculation decreases time needed to complete MLF and positively affected the final wine aroma.

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Chapter 4

General discussion and conclusions

4. General discussion and conclusions

4.1 Concluding remarks and future work

Malolactic fermentation (MLF) is the secondary fermentation that normally occurs after alcoholic fermentation (AF) in most red- and some white wines (Lerm *et al.*, 2011). This process is a decarboxylation reaction whereby L-malic acid is converted into L-lactic acid with the production of carbon dioxide, conducted by lactic acid bacteria (LAB), mostly *Oenococcus oeni* (formerly known as *Leuconostoc oenos*), but nowadays also *Lactobacillus plantarum* (Dicks *et al.*, 1995; Solieri *et al.*, 2010). The most important reasons for conducting MLF are to deacidify the wine, to improve microbial stability by removing malic acid as carbon source as well as to modify wine aroma (Maicas *et al.*, 1999; Liu, 2002; Bartowsky and Borneman, 2011; Knoll *et al.*, 2011). Other LAB genera associated with MLF include *Pediococcus* and *Leuconostoc* (Lonvaud-Funel, 1999; Muñoz *et al.*, 2011). The reason for the predominant use of *O. oeni* in commercial starter cultures today is due to their tolerance to the harsh wine environment, but due to the resilience of *L. plantarum* to the harsh wine environment, it too has been implemented in commercial starter cultures (Du Toit *et al.*, 2011; Lerm *et al.*, 2011). Different inoculation regimes can be used to conduct MLF. They include inoculating the bacteria with the yeast (co-inoculation), mid-AF and after AF (sequential inoculation). There is a realization amongst winemakers about the advantages of inoculated MLF compared to the disadvantages associated with spontaneous MLF. The use of co-inoculation in commercial cellars are also increasing because it allows for earlier completion of fermentation (AF and MLF) that allow the winemaker to better manage and utilize tank space without negatively affecting AF or risking off-flavours (Jussier *et al.*, 2006; Nehme *et al.*, 2010).

The overall objective of this study was to assess the impact of yeast and nutrient additions on the NT 202 Co-Inoculant mixed MLF starter culture (comprised of *O. oeni* and *L. plantarum*) and the aroma compound production in the final wine. This study addressed the limited knowledge on the use of mixed MLF starter cultures used in co-inoculation.

The first aim of this study was to evaluate the impact of different red and white wine yeast strains on the ability of the NT 202 Co-Inoculant compared to commercial starter cultures consisting of only *O. oeni* to conduct MLF during co-inoculation in Chardonnay, Merlot and Shiraz. Malolactic fermentation was unsuccessful in Chardonnay probably due to very high initial acidity, but co-inoculated MLF in Merlot and Shiraz showed earlier completion compared to the un-inoculated MLF controls. Successful MLF using the mixed MLF starter culture was also found by Lerm (2010). A re-evaluation in Chardonnay is advised. This study showed that the compatibility of the yeast strains with the NT 202 Co-Inoculant is different and was grouped into three categories concerning the malic acid degradation of the NT 202 Co-Inoculant, namely inhibitory, neutral and stimulatory towards MLF. Yeast strains that showed positive compatibility with the NT 202 Co-Inoculant include WE 372 and Exotics. The next step was to investigate the

impact of the yeast-bacterial combinations on the aroma compound production in the final wine. Co-inoculated MLF showed positive aroma changes in the red wines and generally MLF led to increased total ester concentrations, especially ethyl lactate and diethyl succinate. This was also found by other studies (Lerm, 2010; Malherbe, 2010; Knoll *et al.*, 2011). The increase in ethyl lactate is attributed to the esterification of ethanol and lactate with increasing concentrations due to AF and MLF, respectively. Ethyl lactate is associated with fruity, buttery and creamy aromas and contributes to better mouthfeel of the wine (Lerm *et al.*, 2010). Diethyl succinate contributes fruity aromas to the wine and is formed via the non-enzymatic esterification of succinic acid, a by-product of microbial α -ketoglutarate metabolism (Peinado *et al.*, 2004; Ugliano and Moio, 2005). The production of total esters, volatile fatty acids and higher alcohols seems to be dependent on yeast- and LAB strain. The NT 202 Co-Inoculant contributed to the monoterpenes produced due to *L. plantarum* in the mix and its β -glucosidase activity. Malolactic fermentation resulted in increased concentrations of diacetyl and acetoin, carbonyl compounds responsible for buttery characters in wine, which correlates with findings of other studies (Lerm, 2010; Malherbe, 2010). The mixed MLF starter culture was able to maintain sufficient microbial populations until completion of MLF with similar MLF rates to that of the commercial *O. oeni* MLF starter culture controls. No sudden or severe decrease in bacterial cell numbers were observed after co-inoculation, which indicates that by-products or metabolites produced by yeasts during co-inoculated MLF, did not have a negative effect on the LAB. It is clear from this study that different LAB strains present in the MLF starter cultures can result in differences in final wine aroma. Further research is still needed to evaluate the yeast-bacterial interactions with different inoculation times in bigger vinification volumes and to include more cultivars subjected to MLF from different wine regions. The change in the associated aroma compound production of such wines during ageing should also be investigated. A complete sensorial evaluation including descriptive analysis will enhance the existing knowledge on aroma modifications associated with MLF. If descriptive sensory data is correlated with consumer-generated sensory and chemical data, it can add valuable information regarding consumer preference with regard to wine style (Malherbe, 2010). The winemaker can then use this as a tool to produce wines fit for a consumer-driven market.

The second aim of this study was to investigate the impact of different wine additives such as yeast- and bacterial nutrients, clarifying agents as well as detoxifying agents on the ability of the NT 202 Co-Inoculant to conduct co-inoculated MLF and to assess their impact on the final wine aroma. This study showed that wine additives used during co-inoculation had no negative or positive impact on MLF and associated aroma compounds. Knowledge on the impact of such wine additives used during co-inoculation and their effect on wine aroma is limited. Therefore further research is needed to investigate the impact of the specific components of these wine additives on MLF conducted with different inoculation regimes, in different cultivars over various vintages.

The final aim of this study was to use multivariate data analysis techniques to investigate underlying trends in the datasets concerning the aroma compound production during co-inoculated MLF with NT 202 Co-Inoculant. Generally this study showed that MLF as well as LAB starter culture used had an effect on the final wine aroma. This was also confirmed by a study done by Lerm (2010).

This study creates various future research projects. These include the investigation of mixed LAB starter cultures in other commonly used South African red grape varieties as well as concentrating on the effect of different inoculation regimes on bacterial performance. The aroma changes associated with the individual LAB strains in the mixed culture need to be investigated and can provide important information concerning the specific contributions of the individual *O. oeni* and *L. plantarum* strains.

This study clearly showed the positive contribution of the mixed MLF starter culture, containing *O. oeni* and *L. plantarum*, on the final wine aroma and its positive compatibility with especially WE 372 as well as Exotics during co-inoculation of yeast strains tested.

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