The production of volatile phenols by wine microorganisms

by

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DECLARATION

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SUMMARY

The production of good quality wine is essential to ensure competitiveness on an international level. Wine quality is usually evaluated for the visual, olfactory and taste characteristics of that specific wine. The winemaking process starts with the grapes in the vineyard followed by oenological practises in the winery until the final wine is bottled. Factors that could influence wine quality include the grape quality from which the wine is made and different techniques used during wine production. Other factors include the presence as well as the interaction between microorganisms found in the grape juice and wine, and the biochemical effect these microorganisms have on certain chemical compounds in the wine. The different microorganisms found in grape juice and wine can either have a negative or positive contribution to the final quality of the wine. During certain stages of the winemaking process the growth and metabolic activity of certain microorganisms is a necessity to produce good wine. During other stages the presence of certain microorganisms can lead to the development of compounds that is regarded as off-flavours and therefore lead to unpalatable wines of low quality.

Yeast strains that naturally present on the grapes and in the winery can also contribute to the final quality of the wine. *Brettanomyces* yeasts are part of the natural flora of winemaking and can drastically influence the aroma characters of a wine through the production of volatile phenols. The general aroma descriptions of volatile phenols include "smoky", "spicy", "barnyard", "animal" and "medicinal". Although some wine drinkers believe that these characters can add to the complexity of a wine, high levels of volatile phenols is mostly regarded as off-flavours and mask the natural fruity flavours of a wine.

With this study we wanted to generate a better understanding of the effect of different winemaking practises on the production of volatile phenols by *B. bruxellensis*. We evaluated the difference in volatile phenol production when *B. bruxellensis* was introduced before or after alcoholic fermentation. We have shown that *B. bruxellensis* could grow and produce volatile phenols during alcoholic fermentation. Results obtained also showed that commercial wine yeast strains could produce the vinyl derivatives that serve as precursors for *Brettanomyces* yeast to produce the ethyl derivatives. The commercial yeast strains differed in their ability to produce vinyl derivatives.

Different malolactic fermentation scenarios were evaluated, namely spontaneous versus inoculated, and with or without yeast lees. Results showed that spontaneous malolactic fermentation had higher volatile phenol levels in the wine than inoculated malolactic fermentation. The treatment with lees reduced the level of volatile phenols, probably due to absorption by yeast cells.

The presence of the phenyl acrylic decarboxylase (*PAD1*) gene and the production of volatile phenols by *S. cerevisiae* commercial yeast strains were evaluated in Shiraz grape juice and in synthetic grape juice. The results indicated that the yeast strains differ in their ability to produce 4-vinylphenol and 4-vinylguaiacol. All the yeast strains tested had the *PAD1* gene.

We also evaluated the presence of the phenolic acid decarboxylase (*padA*) gene and the ability of different lactic acid bacteria strains to produce volatile phenols in synthetic wine media. Although some of these strains tested positive for the phenolic acid decarboxylase gene most of them only produced very low levels of volatile phenols.

This study made a valuable contribution on the knowledge about the effect of *Brettanomyces* yeast on the volatile phenol content of red wines during different stages of the winemaking process and when applying different winemaking practices. It also showed the effect between *Brettanomyces* yeast and other wine microorganisms and the possible influence it could have on the final quality of wine. Research such as this can therefore aid the winemaker in making certain decisions when trying to manage *Brettanomyces* yeast spoilage of wines.

OPSOMMING

Die produksie van hoë gehalte wyn is essensieel om te verseker dat wyne mededingend op die internasionale mark is. Wynkwaliteit word gewoonlik op visuele, olfaktoriese en smaak eienskappe geëvalueer. Die wynmaakproses begin met die druiwe in die wingerd, gevolg deur die wynkundige praktyke in die kelder totdat die finale wyn gebottelleer word. Faktore wat wynkwaliteit beïnvloed, sluit in die kwaliteit van die druiwe waarvan die wyn gemaak word en verskillende tegnieke wat gedurende wynproduksie gebruik word. Ander faktore is die teenwoordigheid van en interaksie tussen mikroörganismes wat in druiwesap en wyn voorkom, sowel as die biochemiese uitwerking van hierdie mikroörganismes op chemiese komponente in die wyn. Die verskillende mikroörganismes wat in druiwesap en wyn voorkom, kan egter ‘n negatiewe of positiewe effek op die finale wynkwaliteit hê. Die groei van sekere mikroörganismes en hul metaboliese aktiwiteit gedurende sekere stadiums in die wynmaakproses is noodsaaklik vir die produksie van goeie wyn. Gedurende ander stadiums kan die teenwoordigheid van sekere mikroörganismes lei tot die ontwikkeling van verbindings, wat as afgeure beskou word, asook aanleiding gee tot ondrinkbare wyne met 'n lae gehalte.

Gisrasse wat natuurlik op druiwe en in die kelder voorkom, kan ook tot die finale wynkwaliteit bydra. *Brettanomyces*-giste is ook deel van die natuurlike flora van die wynmaakproses en kan deur die produksie van vlugtige fenole ‘n drastiese invloed op die aroma-eienskappe van ‘n wyn hê. Die algemene beskrywing van vlugtige fenole sluit in "rokerig", "speseryagtig", "perdestal", "dieragtig" en "medisinaal". Alhoewel sommige wyndrinkers glo dat hierdie eienskappe tot die wynkompleksiteit kan bydra, word hoë konsentrasies van vlugtige fenole meestal beskou as afgeure wat die vrugtige aroma van wyne masker.

Die doel van hierdie studie was om meer insig te verkry oor die effek van verskeie wynmaakpraktyke op die produksie van vlugtige fenole deur *B. bruxellensis*. Die verskil in vlugtige fenoolproduksie is geëvalueer wanneer *B. bruxellensis* voor en na alkoholiese fermentasie geïnokuleer word. Dit is bewys dat *B. bruxellensis* gedurende alkoholiese fermentasie kan groei en vlugtige fenole produseer. Die resultate het ook aangedui dat kommersiële wyngisrasse vinielderivate, wat as voorlopers vir die produksie van etielderivate deur *Brettanomyces*-giste dien, produseer. Die kommersiële gisrasse het ook gevarieer in hul vermoë om vinielderivate te produseer.

Verskillende appelmelksuurgistingspraktyke is ook geëvalueer, naamlik spontane *versus* geïnokuleerde, met of sonder gismoer, praktyke. Resultate het gewys dat spontane appelmelksuurgisting oor die algemeen tot hoër konsentrasies vlugtige fenole in wyn gelei het as dit met geïnokuleerde appelmelksuurgisting vergelyk word. Die behandelings met gismoer het ook oor die algemeen tot laer vlakke vlugtige fenole gelei, wat moontlik aan die absorpsie deur gisselle toegeskryf kan word.

Die teenwoordigheid van die fenielakriliese suurdekarboksilase-geen en die produksie van vlugtige fenole deur verskillende kommersiële *S. cerevisiae*-gisrasse is in

Shiraz- en sintetiese druiwesap geëvalueer. Resultate het aangedui dat gisrasse varieer in hul vermoë om 4-vinielfenol en 4-vinielguajakol te produseer. Al die gisrasse het positief getoets vir die teenwoordigheid van die fenielakriliese suurdekarboksilase-geen.

Die teenwoordigheid van die fenoliese suurdekarboksilase-geen en die produksie van vlugtige fenole deur verskeie melksuurbakterierasse in sintetiese wynmedia is ook geëvalueer. Alhoewel sekere rasse positief getoets het vir die fenoliese suurdekarboksilase-geen, het die meeste van hulle baie lae vlakke vlugtige fenole geproduseer.

Hierdie studie het ‘n waardevolle bydrae gelewer tot die kennis van *Brettanomyces*-giste op die vlugtige fenool-inhoud van rooiwyne gedurende verskillende stadiums van die wynmaakproses en wanneer verskillende wynmaaktegnieke toegepas word. Dit het ook die effek tussen *Brettanomyces*- en ander wynmikroörganismes, asook die moontlike invloed wat dit op die finale wynkwaliteit kan hê, aangedui. Navorsing soos hierdie kan wynmakers help om oordeelkundige besluite te neem ten einde *Brettanomyces*-bederf van wyne beter te bestuur.

BIOGRAPHICAL SKETCH

Lisha Nelson was born on 2 September 1982, attended Slot van die Paarl Primary School and matriculated at Paarl Gymnasium High School in 2000. Lisha obtained a BScAgric degree in Viticulture and Oenology in 2004 at the University of Stellenbosch. In 2006 she enrolled for an MScAgric degree at the same University.

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PREFACE

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

Chapter 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

Chapter 2 **LITERATURE REVIEW**

The production of volatile phenols by wine microorganisms

Chapter 3 **RESEARCH RESULTS**

Investigating the influence *Saccharomyces cerevisiae* and malolactic fermentation on the production of volatile phenols by *Brettanomyces bruxellensis*

Chapter 4 **RESEARCH RESULTS**

The production of volatile phenols by *Saccharomyces cerevisiae* wine yeast strains and lactic acid bacteria associated with wine

Chapter 5 **GENERAL DISCUSSION AND CONCLUSIONS**

CONTENTS

Chapter 1

GENERAL INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Although wine and winemaking date back to the time of ancient Persia and Egypt, the skill of winemaking today has developed into a complex study of wine microbiology and chemistry. The art of winemaking is therefore the result of biological and chemical interactions that take place between grape juice constituents and different micro-organisms (Fleet, 2003).

Currently extensive research is being conducted on the origins of wine aroma and flavour. Even though a great deal of the wine flavour is derived from the grape, not all is evident in the grape berry prior to the vinification process. Part of wine flavour also results from the transformation of non-volatile (flavourless) grape metabolites into volatile aroma compounds by wine microorganisms. Microorganisms that are usually associated with winemaking include yeasts, which are responsible for alcoholic fermentation and lactic acid bacteria (LAB) for malolactic fermentation (MLF). It is very important that the desired microorganisms should dominate during the winemaking process at the right stages to ensure a good quality wine (Fleet, 1993, 2003; Swiegers *et al*., 2005).

During alcoholic fermentation yeasts convert grape sugar into alcohol, but also produce and excrete metabolites, as well as undergo autolysis (Fleet, 1993; Lambrechts & Pretorius, 2000; Fleet, 2003; Swiegers *et al*., 2005; Swiegers & Pretorius, 2005; Jolly *et al*., 2006). These metabolic compounds can contribute to the flavour, mouth feel and quality of the wine. Although *Saccharomyces cerevisiae* yeasts are predominantly associated with alcoholic fermentation, certain non-*Saccharomyces* yeasts can also modulate wine flavour and aroma (Swiegers *et al*., 2005). These include yeast of the genera *Brettanomyces* (and its sexual counterpart *Dekkera*), *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora* (and its asexual counterpart *Kloeckera*), *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* (Pretorius *et al*., 1999). Some of these yeast species can act as spoilage organisms during the winemaking process and maturation of the wine. Yeast spoilage typically includes slight cloudiness or haziness in bottled wines, sediment and chemical off-flavours in wines (Loureiro & Malfeito-Ferreira, 2003).

The yeast species *Brettanomyces bruxellensis* is regarded as spoilage microorganism commonly associated with wine and other fermentable beverages (Van der Walt, 1964; Thurston & Tubb, 1981; Morrissey *et al*., 2004; Silva *et al*., 2004). Wine contamination by *B. bruxellensis* include haziness or turbidity (van der Walt & van Kerken, 1958), the formation of mousy off-flavours (Heresztyn, 1986; Grbin & Henschke, 2000), high levels of acetic acid (Ciani & Ferraro, 1997) and especially the production of volatile phenols (Chatonnet *et al*., 1992). The volatile phenols

predominantly responsible for the phenolic off-flavour of wine include 4-vinylphenol (4-VP), 4-vinylguaiacol (4-VG), 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) with the latter two currently used as indicators of *Brettanomyces* yeast activity in wine (Chatonnet *et al*., 1992). More recently 4-ethylcatechol has been mentioned to be present in wine and can therefore also influence wine aroma (Hesford *et al*., 2004). General aroma descriptors of wines with *Brettanomyces* yeast contamination and elevated levels of volatile phenols include 'medicinal', 'Band-aid', 'barnyard', 'leathery', 'horsy', 'sweaty', 'wet dog', 'smoky' and 'spicy' (Chatonnet *et al*., 1992; Rodrigues *et al*., 2001).

Volatile phenols found in wine are formed by the microbial transformation of hydroxycinnamic acids naturally present in grapes (Boulton *et al*., 1996). These hydroxycinnamic acids are usually bound in the form of tartaric acid esters from which free hydroxycinnamic acids are released by the process of hydrolysis (Dugelay *et al*., 1993; Lao *et al*., 1997). The conversion of hydroxycinnamic acids, including *p*-coumaric acid, ferulic acid and caffeic acid, into volatile phenols involves a two-step pathway. During the first step the hydroxycinnamate decarboxylase enzyme converts the hydroxycinnamic acids into the vinyl derivatives (Chatonnet *et al*., 1992; Edlin *et al*., 1995). Thereafter vinyl phenol reductase reduces the vinyl derivatives (4-VP and 4-VG) into ethyl derivatives (4-EP and 4-EG) (Dias *et al*., 2003).

It is very important for winemakers to ensure the competitiveness of their products on the international market. As the contamination of wine by *Brettanomyces* yeast through the production of volatile phenols can be detrimental to wine quality, it can lead to great economical losses for a winery. This is predominantly the reason for the amplified interest and focus of wine microbiologists on the potential spoilage capacity of these yeasts. It was previously considered that *Brettanomyces* yeast spoilage occurred mostly during aging of wines in barrels (Fugelsang, 1997; Licker *et al.,* 1998). However, according to literature these yeasts can come from grape bunches in the vineyard and grow and produce spoilage products during early stages of the winemaking process. It is therefore important for winemakers to be aware that *Brettanomyces* yeast can cause spoilage during alcoholic fermentation (Wright & Parle, 1973; Licker *et al*., 1998; Renouf & Lonvaud-Funel, 2007).

Wine microorganisms other than *Brettanomyces* yeasts also have the ability to produce certain volatile phenols during the winemaking process that could lead to the depreciation of wine quality. The production of volatile phenols by *Saccharomyces cerevisiae* (Chatonnet *et al*., 1993), other non-*Saccharomyces* yeasts (Shinohara *et al*., 2000; Dias *et al*., 2003; Barata *et al*., 2006) and some lactic acid bacteria strains could also influence the aroma characteristics of wine (Cavin *et al*., 1993; Chatonnet *et al*., 1995; Couto *et al*., 2006).

There is only a limited amount of research on the spoilage capacity of *Brettanomyces* yeast during the early stages of the winemaking process as well as the interaction of these yeasts with other wine microorganisms. The generation of such information can be directly beneficial to the winemaker and the incorporation of this data

into certain winemaking practises can aid during the implementation of control strategies to limit the spoilage of wines by *Brettanomyces* yeasts.

1.2 PROJECT AIMS

This study forms an integral part of a larger research project on wine spoilage caused by microorganisms that is being conducted at the Department of Viticulture and Oenology and the Institute for Wine Biotechnology at Stellenbosch University. The overriding goal of this study was to generate a better understanding of the effect of different winemaking practises on the production of volatile phenols by *B. bruxellensis*. The possibility of other wine microorganisms to produce volatile phenols was also evaluated. The specific aims of the study were as follows:

(i) to evaluate the difference in volatile phenol production when *B. bruxellensis* was introduced before alcoholic fermentation into grape juice or after alcoholic fermentation into wine;

(ii) to assess the impact that different *S. cerevisiae* commercial wine yeast strains will have on the volatile phenol levels produced by *B. bruxellensis*;

(iii) to investigate different malolactic fermentation scenarios and determine the impact thereof on the volatile phenol production by *B. bruxellensis*;

(iv) to evaluate the ability of different commercial wine yeast strains to produce volatile phenols in grape juice and in synthetic grape juice;

(v) to screen different *S. cerevisiae* commercial wine yeast strains for the presence of the phenyl acrylic acid decarboxylase (*PAD1*) gene with PCR;

(vi) genetic screening of different strains of lactic acid bacteria for the presence of the phenolic acid decarboxylase (*padA*) gene; and

(vii) to assess the production of volatile phenols by lactic acid bacteria strains in synthetic wine media.

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Chapter 2

LITERATURE REVIEW

The production of volatile phenols by wine microorganisms

LITERATURE REVIEW

The production of volatile phenols by wine microorganisms

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This work reviews the production of volatile phenols by various wine microorganisms from the hydroxycinnamic acid precursors, with a focus on *Brettanomyces*/*Dekkera* yeasts. The factors that influence the growth and volatile phenol production of these yeasts are also discussed. Furthermore the possible prevention and control of *Brettanomyces*/*Dekkera* contamination of wines are discussed. The interactions between *Brettanomyces*/*Dekkera* yeasts and other wine microorganisms and the effect thereof on the final volatile phenol content of wines are reviewed.

2.1 INTRODUCTION

Quality control during the winemaking process ensures that the final product is competitive on the international market and of a sufficient quality standard. The quality of a wine is usually evaluated on its visual, olfactory and taste characteristics. There are several winemaking practices and production techniques that can have an influence on the final quality of the wine, considering that they can alter the chemical and microbial status of the wine and consequently influence the taste and aroma of a specific wine. The improved quality requirement by wine consumers extended the range of potential spoilage effects and reduced the acceptance of characteristics formally not identified as contributing negatively to the quality of wine. These defects include slight cloudiness or haziness in bottled wines, sediment, various off-flavours and phenolic taint in wines, to name just a few (Loureiro & Malfeito-Ferreira, 2003).

Various microorganisms are involved during the winemaking process and their growth and metabolism can have an immense impact on the quality of a wine. Wine can therefore be described as the product of a complex interaction between different microorganisms and chemical constituents of grape juice. Yeast is responsible for the alcoholic fermentation process during which grape sugars are converted into ethanol and carbon dioxide. This process is mostly dominated by *Saccharomyces cerevisiae* yeast, but non-*Saccharomyces* yeast can also have an influence during fermentation (Jolly *et al*., 2006). Non-*Saccharomyces* yeast genera associated with winemaking include *Brettanomyces* (and its sexual counterpart *Dekkera*), *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, (and its asexual counterpart *Kloeckera*), *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* (Pretorius *et al*., 1999).

Yeasts can have an affect on wine aroma, flavour, mouth-feel, colour and chemical composition by the production and excretion of metabolites during their growth and autolysis (Swiegers *et al*., 2005). Lactic acid bacteria (LAB) and acetic acid bacteria (AAB) also play a very important role during the winemaking process. Acetic acid bacteria are considered to be spoilage microorganisms due to the production of excessive amounts of acetaldehyde and acetic acid (Drysdale & Fleet, 1988). The four genera of LAB associated with wine include *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* (Davis *et al*., 1988). *Oenococcus oeni* is the main LAB responsible for the process of malolactic fermentation (MLF) during which malic acid is converted into lactic acid resulting in the de-acidification and microbial stability of wines (Wibowo *et al*., 1985). LAB can also modify certain compounds that could have an influence on the sensory properties of wine consequently having a detrimental affect on the quality of the final product (Henick-Kling, 1993; Lonvaud-Funel, 1999).

Spoilage microorganisms are defined as being unwanted at a particular place or time and produce metabolites that negatively contribute to the taste, flavour or aroma of the final product. However, controversial opinions exist between individuals as to whether some of these metabolites are perceived as detrimental or beneficial to wines. This is especially true for the production of volatile phenols by *Brettanomyces*/*Dekkera* yeasts. Although the excessive production of 4-ethylphenol by *Brettanomyces* in red wines (above 620 µg/L) is regarded as spoilage, a concentration below this threshold can be regarded as contributing to the complexity of wine aroma (Chatonnet *et al*., 1992). The name *Dekkera* is often used as an interchangeable name for *Brettanomyces* and based on their molecular similarities these yeasts belong to related, but different genera (Harris *et al*., 2008). *Dekkera* refers to the sexual or sporulating form of the yeast, and *Brettanomyces* the asexual or non-sporulating form (Boulton *et al*., 1996; Arvik & Henick-Kling, 2002).

Contamination of wines by *Brettanomyces* yeast is becoming an increasing concern in the wine industry causing great economical losses and these yeasts are regarded as the main spoilage microorganisms in wines today affecting especially premium red wines matured in oak barrels (Loureiro & Malfeito-Ferreira, 2003; Silva *et al*., 2004). Currently the possible contamination of red wines by *Brettanomyces bruxellensis* is the most dreaded by winemakers. Reasons for this is that the production of high concentrations of volatile phenols especially 4-ethylphenol and 4-ethylguaiacol, leads to an excessive "*Brettanomyces*" character and masks the fruitiness of wines (Romano *et al*., 2008).

Although *Brettanomyces* yeasts are considered to be the main producers of volatile phenols, other wine microorganisms also have the ability to produce volatile phenols that could lead to the depreciation of wine quality. These include *Saccharomyces cerevisiae* (Chatonnet *et al*., 1993) and non-*Saccharomyces* yeasts (Shinohara *et al*., 2000; Dias *et al*., 2003a; Barata *et al*., 2006) that have been shown to produce volatile phenols. Some LAB strains are also able to produce volatile phenols that could

influence the aroma characteristics of wine (Cavin *et al*., 1993; Chatonnet *et al*., 1995; Couto *et al*., 2006).

In addition to volatile phenols, *Brettanomyces* yeasts can also produce other compounds that could have a negative impact on wine quality. These include the production of excessive amounts of acetic acid and ethyl acetate (Boulton *et al*., 1996; Ciani *et al*., 1997; Harris *et al*., 2008), the formation of tetrahydropyridins (Heresztyn *et al*., 1986a; Grbin & Henschke, 2000; Snowdon *et al*., 2006) and isovaleric acid (Renouf & Lonvaud-Funel, 2007). *Brettanomyces* yeasts growth can also visually influence the quality by causing haziness in wines (van der Walt & van Kerken, 1958).

This review will focus on the production of volatile phenols by wine microorganisms with the biggest emphasis on *Brettanomyces* spp. The factors affecting the growth of these yeasts and their capability to produce volatile phenols are described. Furthermore, the possible techniques for the prevention or control of these yeasts will be discussed.

2.2 THE EFFECT OF VOLATILE PHENOLS ON WINE QUALITY

The production of volatile phenols by microorganisms in wine can have a influence on the aroma of the wine. The volatile phenols associated with wine include 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol (Chatonnet *et al*., 1992). Recently, the presence of 4-ethylcatechol has been reported in wines and this compound can also influence wine aroma (Hesford *et al*., 2004). Elevated levels of volatile phenols can cause a phenolic taint in wines and in severe cases the wine can be described as having an off-flavour. The general aroma description of wines with high levels of volatile phenols includes phenolic, medicinal, horse sweat, leather, smoky etc. (Chatonnet *et al*., 1992; Rodrigues *et al*., 2001). Ethylphenols can affect the wine quality negatively even if it is present at low quantities in red wines. For example concentrations over 425 µg/L, with a 1:10 ratio of 4-ethylguaiacol to 4-ethylphenol, produce animal-like, phenolic and barnyard characters in red wines, depending on the wine style (Chatonnet *et al*., 1995). Chatonnet *et al*. (1992) found that elevated levels of volatile phenols could appear at different winemaking stages, but usually occurs when wines are aged in oak barrels and especially during the re-use of older barrels. The sensory thresholds and aroma influence of the above-mentioned volatile phenols are summarised in **Table 2.1**.

Extensive research done by the brewing industry found that the presence of vinyl derivatives in beers could lead to the formation of off-flavours. It was also found that certain yeasts that perform the fermentation of beer (Steinke & Paulson, 1964; Thurston & Tubb, 1981) and wine have the ability to produce 4-vinylphenol and 4-vinylguaiacol (Chatonnet *et al*., 1993, 1995). According to Chatonnet *et al*. (1993) only white wines contain substantial quantities of vinyl-derivatives and if that exceeds the combined threshold value of 725 µg/L (1:1 of 4-vinylguaiacol and 4-vinylphenol) it can be responsible for phenolic and pharmaceutical characteristics.

TABLE 2.1
Concentrations, aroma thresholds and description of volatile phenols commonly found in red wines (Curtin *et al*., 2005).

Compound	Concentration in red wine (µg/L)	Aroma threshold (µg/L)	Aroma descriptor
4-ethylphenol	118-3696	440*/600**	Phenol, Band-aid, medicinal, barnyard
4-ethylguaiacol	1-432	33*/110**	Spice, clove
4-ethylcatechol	27-427	30-60**	Horse-like
4-Vinylphenol	8.8-43	20***	Phenol, medicinal
4-Vinylguaiacol	0.2-15	10*	Clove-like

*Model wine, **red wine, ***water.

2.3 SYNTHESIS OF VOLATILE PHENOLS

Volatile phenols originate from precursors naturally present in grapes known as phenolic acids or hydroxycinnamic acids. Hydroxycinnamic acids are usually present in low concentrations in grapes, normally esterified with tartaric acid and include caftaric acid, coutaric acid and fertaric acids (Boulton *et al*., 1996; Lao *et al*., 1997). Other reported esters of hydroxycinnamic acids include: ethyl-esters, glucose-esters and anthocyanin coumarates (Curtin *et al*., 2005). The free form of hydroxycinnamic acids, including: *ρ*-coumaric acid, ferulic acid and caffeic acid can be released by certain cinnamoyl esterase activities during the winemaking process (Dugelay *et al*., 1993; Stead, 1995).

Phenolic acids in plant cells serve as a natural toxin against the growth of undesirable microorganisms (Barthelmebs *et al.,* 2000a, b). The free form of hydroxycinnamic acids can be modified by certain microorganisms to less toxic compounds. The hydroxycinnamate decarboxylase enzyme, produced by various microorganisms, is responsible for the transformation of the free hydroxycinnamic acids thereby resulting in the elimination of the antimicrobial effect (Stead, 1995).

The formation of volatile phenols (**Figure 2.1**) involves the action of hydroxycinnamate decarboxylase on *ρ*-coumaric acid, ferulic acid or caffeic acid converting it into the vinyl-derivatives (Edlin *et al*., 1995). The subsequent action of vinylphenol reductase reduces the hydroxystyrenes (vinylphenols) to their ethyl derivative forms (Dias *et al*., 2003a).

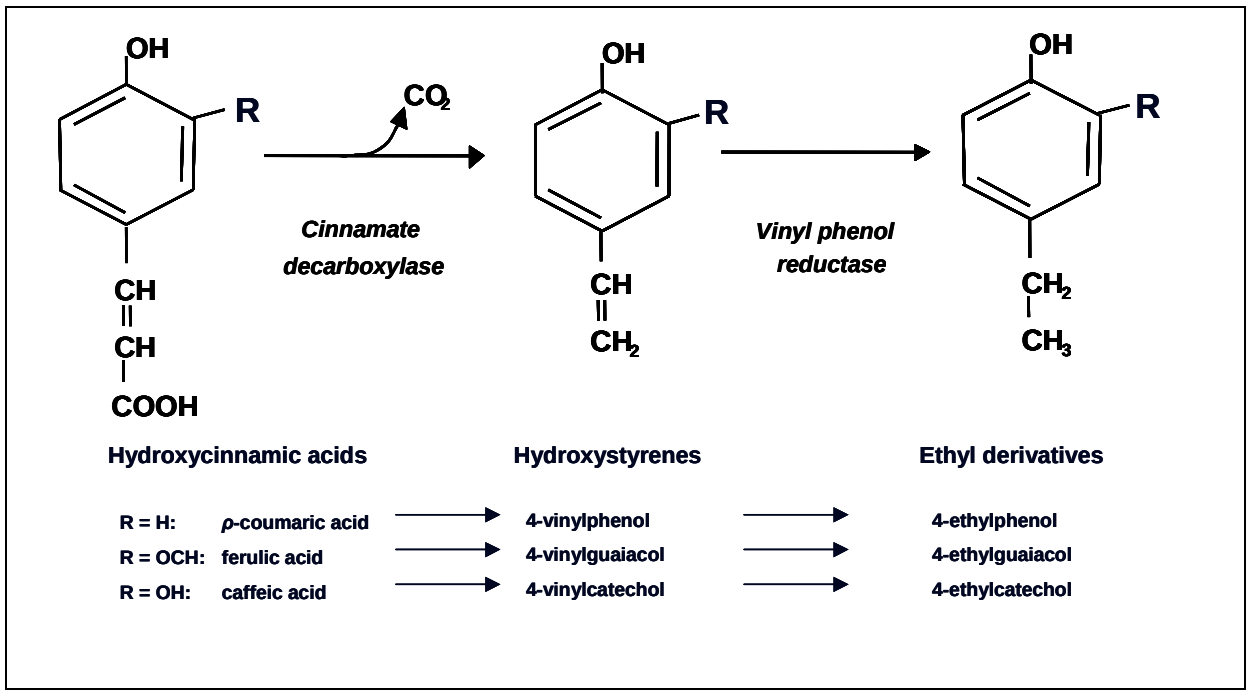

FIGURE 2.1
The formation of volatile phenols from their hydroxycinnamic acid precursors.

2.4 THE PRODUCTION OF VOLATILE PHENOLS BY *BRETTANOMYCES/ DEKKERA*

2.4.1 *BRETTANOMYCES*: ORIGIN AND OCCURRENCE

In 1904 Claussen stated that after the primary fermentation of English stock beer a secondary fermentation was performed. This secondary fermentation was conducted by inoculating the wort with a non-spore forming non-*Saccharomyces* yeast strain. The effect of this secondary fermentation led to the production of flavour compounds that became very typical of strong British beers (Licker *et al*., 1998; Arvik & Henick-Kling, 2002). The name *Brettanomyces* was chosen, because of the close relation of this yeast with the British brewing industry considering that “Brettano” means British brewer and “myces” means fungus. Hence, *Brettanomyces* means British brewing fungus (Licker *et al*., 1998).

Literature has indicated that *Brettanomyces* species have been associated with various fermented products including wine, cider, beer, kombucha and kefyr (Van Der Walt, 1964; Thurston & Tubb, 1981; Licker *et al*., 1998; Arvik & Henick-Kling, 2002; Morrissey *et al*., 2004; Suárez *et al*., 2007). According to Arvik and Henick-Kling (2002) *Brettanomyces* spp. is ubiquitous and can be found in all wine producing countries around the world. *Brettanomyces* spp. have been reported in wines from winemaking regions including Germany, France, South Africa, Italy, Brazil, Uzbekistan, Spain, Portugal, New Zealand, Great Britain and the United States (Licker *et al.,* 1998). In an extensive and more recent study by Curtin *et al*. (2007) *Dekkera bruxellensis* yeasts were isolated from wines from all over the Australian winemaking regions.

Previously winemakers believed that insufficient cellar hygiene was the only cause of *Brettanomyces* yeast contamination. However, Renouf and Lonvaud (2004) stated that despite extreme hygiene practices contamination still occurred in French wineries. They suggested that an origin upstream of the wineries should be considered. Although the presences of *Brettanomyces* spp. on grape bunches have been speculated, researchers have found it extremely difficult to isolate *Brettanomyces* yeasts from grapes in the vineyard (Arvik & Henick-Kling, 2002; Renouf & Lonvaud-Funel, 2007; Suárez *et al.,* 2007). Reasons for this might include the fact that they require a reasonably complex exogenous nutrient supplementation and therefore their growth would be limited on undamaged grape berries (Fugelsang, 1997). The isolation of *Brettanomyces* spp. also becomes very difficult in a complex mixture of more dominating or faster growing microorganisms (Prackitchaiwattana *et al*., 2004). Recently a study by Renouf and Lonvaud-Funel (2007) investigated the presence of *B. bruxellensis* on the surface of grape berries. They developed an enrichment medium (named EBB) for *B. bruxellensis* that enabled them to overcome the detection limit of molecular methods (species-specific PCR, ITS-RFLP PCR, PCR-DGGE) used during their study. They were the first to clearly show the presence of *B. bruxellensis* in several vineyards at different grape ripening stages after veraison. They concluded that the grape berry is the primary source of *B. bruxellensis* contamination and sufficient control of this yeast should start in the vineyard.

Brettanomyces/Dekkera yeasts are seldom found during alcoholic fermentation, although they have been isolated together with *Saccharomyces* yeasts (Wright & Parle, 1973). A possible explanation for this is that *Brettanomyces/Dekkera* yeasts display a metabolism during which alcoholic fermentation by these yeasts is inhibited under anaerobic conditions (Ciani & Ferraro, 1997). This is called the Custers or negative Pasteur effect where by the progression from aerobic to anaerobic conditions detains the growth of *Brettanomyces/Dekkera* yeasts (Suárez *et al*., 2007). These yeasts have been isolated from fermenting grape musts from various countries including France, Germany, Italy, South Africa, Uzbekistan, New Zealand and Spain (Licker *et al.,* 1998). During a survey 1973 on the occurrence of *Brettanomyces* in the New Zealand wine industry, *Brettanomyces* was isolated from 6 out of the 15 wineries investigated during fermentation (Wright & Parle, 1973). This survey also suggested that the must of white grapes were more commonly infected with these yeasts and had higher population densities than red must.

According to literature, *Brettanomyces* yeasts are more commonly detected in the winery, on winery equipment and particularly oak barrels (Ciani and Ferraro, 1997; Fugelsang, 1997; Suárez *et al*., 2007). Furthermore, it is suggested that *Brettanomyces* yeast contaminations increase with the use of contaminated and poorly sanitised winery equipment and barrels (Fugelsang, 1997).

It has been suggested that fruit flies (*Drosophila melanogaster*) and bees can facilitate the spread of yeast species, including *Brettanomyces* within a winery (Licker *et al*., 1998). Yeasts can attach themselves to the body, legs and wings of insects and

can therefore be spread without direct contact with the contaminated areas (Licker *et al*., 1998; Martorell *et al*., 2006). Other possible vectors that may host several yeast species in the vineyard include earthworms, snails, spiders, wasps, crane flies, aphids, manure flies and ants. Although other yeasts have been isolated from these insects no specific mention was made about the isolation of *Brettanomyces* (Licker *et al*., 1998). Connell *et al*. (2002) developed a technique for the rapid detection of *Brettanomyces* spp. from winery air samples based on peptide nucleic acid analysis and successfully identified *Brettanomyces* yeasts from various places around the winery. This suggests the possibility of airborne contamination that could lead to the spread of *Brettanomyces* yeast infection in a winery.

Wooden cooperages however have been stated to be the most common source of *Brettanomyces* contamination in a winery (Fugelsang, 1997; Licker *et al.,* 1998). In 1990 it was reported that *Brettanomyces* infection of various wineries in the United States could be traced to the purchase of oak barrels previously used for the production of red wines (Licker *et al.,* 1998). In addition it has been shown that it is virtually impossible to sterilise *B. bruxellensis* infected barrels (Pollnitz *et al*., 2000). All sterilisation methods tested including washing and rinsing with sulphated water, shaving and firing or ozone treatment could not effectively sterilise infected barrels (Pollnitz *et al*., 2000; Arvik & Henick-Kling, 2002). Some species of *Brettanomyces* yeasts are capable of producing the enzyme β-glucosidase. This enzyme allows it to assimilate cellobiose, which is an alpha-linked disaccharide of glucose, a fragment of cellulose extracted from the wood (Boulton *et al.,* 1996; Suárez *et al*., 2007). There are some indications that new barrels have a stimulating effect on *Brettanomyces* yeast growth. Considering that new barrels contain higher amounts of cellobiose than used barrels they have the potential to sustain higher *Brettanomyces* yeast populations (Boulton *et al.,* 1996). It has been suggested that increased *Brettanomyces* yeast infection of wines during barrel maturation is due to favourable growth factors including the presence of oxygen and nutrients such as residual glucose and nitrogen in aging wines (Licker *et al.,* 1998; Aguilar-Uscanga *et al*., 2003; Oelofse & Du Toit, 2006).

2.4.2 *BRETTANOMYCES*: MORPHOLOGY AND PHYSIOLOGY

Brettanomyces spp. has a very unique characteristic considering that cells in one colony can consist of diverse morphologies (Kurzmann & Fell, 1998). The most characteristic cell morphology is an ogival shape, giving the cells an outline of a pointed arch. Examination of these cells with a microscope shows that the flat ends resulted from distal, adjacent bud scars (Boulton *et al.,* 1996). Other cell morphologies for *Brettanomyces* spp. include spherical, ellipsoidal, cylindrical, boat-shaped and elongated. Considering that these shapes could represent a variety of other wine related yeasts it is very difficult to identify *Brettanomyces* spp. by simple microscopic techniques. **Figure 2.2** illustrates some of the cell morphologies of *Brettanomyces* spp. yeasts. In a study by Millet and Lonvaud-Funel (2000) the size of *Brettanomyces* spp. was found to vary between (5-8) x (3-4) µm in wines. It was also suggested that these

yeasts could change in size, becoming smaller during unfavourable conditions. The identification and isolation of these yeasts should therefore be verified by molecular detection methods and not based on morphological characteristics alone (Millet & Lonvaud-Funel, 2000).

Previous studies have shown inconsistency with regard to the growth parameters of different *Brettanomyces* yeast strains. The different growth conditions and different strains used to evaluate some of these parameters could be the explanation for the contradictory results (Conterno *et al*., 2006). *Brettanomyces* yeasts can utilize various carbon sources including ethanol and therefore the growth of these yeasts may well be supported in wine with small quantities of residual sugar (Chatonnet *et al*., 1995; Dias *et al*., 2003b). Nitrogen sources utilised by most *B. bruxellensis* strains include ammonium, proline, arginine and nitrate (Conterno *et al*., 2006). Conterno *et al*. (2006) also indicated during their study that all the *B. bruxellensis* strains they tested required biotin and thiamine for their growth.

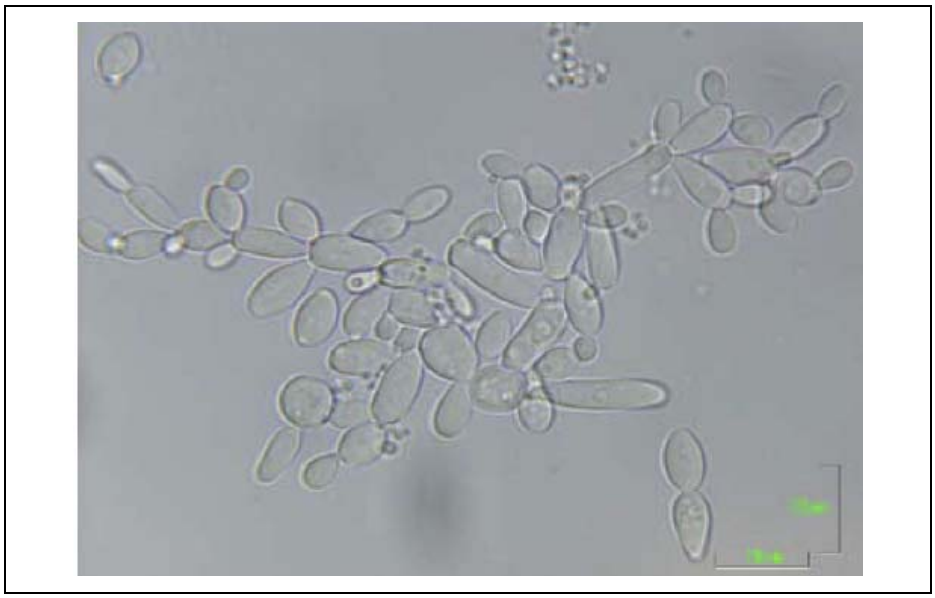

FIGURE 2.2
Optical microscope image of a *Brettanomyces* yeast strain (1000x enlarged) (Suárez *et al.,* 2007).

2.4.3 *BRETTANOMYCES*: FACTORS AFFECTING THEIR GROWTH AND PRODUCTION OF SPOILAGE PRODUCTS

2.4.3.1 Carbon and energy sources

It has been stated that although various residual sugars are found in wines after alcoholic fermentation, *B. bruxellensis* is only capable of fermenting glucose, fructose, galactose and trehalose (Chatonnet *et al*., 1995). Chatonnet *et al*. (1995) investigated the influence of *Brettanomyces* spp. on the ethylphenol content of red wines. They found that *Brettanomyces* spp. growth could be supported by the presence of very low quantities of residual sugars. Considering that *Brettanomyces* yeast is also capable of growing under anaerobic conditions, dry wines (residual sugar levels under 5 g/L) holds greater risk for the development of high levels of ethylphenols. Chatonnet *et al*. (1995)

also showed that *Brettanomyces* yeast development in dry red wines results in a further reduction in sugar levels, especially for trehalose. In a more recent study Conterno *et al*. (2006) found that most of the 35 *Brettanomyces* yeast isolates tested could grow on the monosaccharides glucose, fructose, galactose and the disaccharides sucrose, maltose, cellobiose and trehalose. The growth of most of the isolates tested was not supported by other sugars including arabinose, lactose and raffinose. The growth of these isolates was also not supported by sugar alcohols adonitol, glycerol and mannitol. They also found that 25% of the isolates could grow on ethanol as a sole carbon source and less than 10% grew on soluble starch. A few of the isolates could grow on the organic acids such as lactate, malate, succinate and citrate, but not on tartrate.

Chatonnet *et al*. (1995) also investigated the relationship between the synthesis of ethylphenols and the fermentation of residual sugars during aging of red wines in different barrels in the same cellar. It was found that as the amount of sugar fermented increased, the concentration of ethylphenols increased. In **Figure 2.3** it is clear that after 275 mg/L of the residual sugar is fermented the concentration of ethylphenols formed is equal to its preference threshold of 425 µg/L. At 300 mg/L fermented sugar the ethylphenol content is high above the perception threshold of 600 µg/L (wine in barrel).

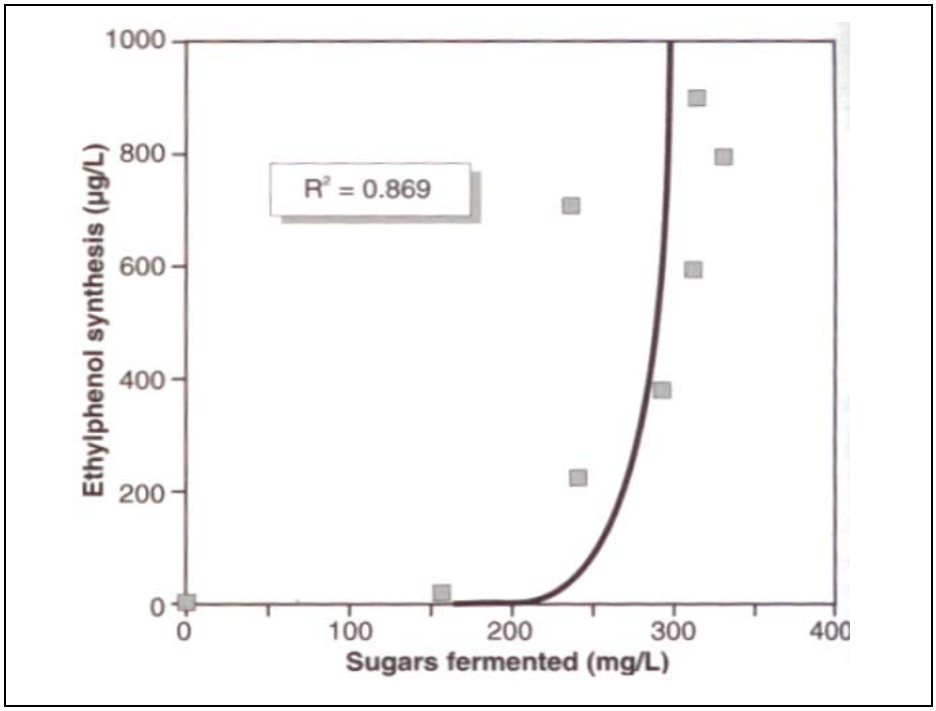

FIGURE 2.3
The exponential regression curve of the relationship between the synthesis of ethylphenols and fermentation of residual sugars (glucose + fructose + galactose + trehalose) by *Brettanomyces* yeasts during the aging of red wines in several barrels in the same cellar (Chatonnet *et al*., 1995).

Dias *et al*. (2003b) found that high conversion rates of *ρ*-coumaric acid to 4-ethylphenol by *B. bruxellensis* only occurred when glucose and ethanol was used as carbon and energy source. Contrary to the results found by Chatonnet *et al*. (1995) they found that the conversion rate of *ρ*-coumaric acid to 4-ethylphenol in the presence of

trehalose is very low. They also found that fermentable sugars less than 2 g/L were not a restriction on the production of high levels of 4-ethylphenol by *B. bruxellensis*.

2.4.3.2 Precursors

The formation of volatile phenols in wine will depend on the amount of the precursor present from which it is formed (Suárez *et al*., 2007). As previously stated the production of 4-ethylphenol from phenolic acids is the result of the consecutive action by two enzymes (**Figure 2.1**).

It is therefore clear that the amount of the hydroxycinnamic acid precursor present in grapes can have an influence on the final volatile phenol concentration in wines. Grape varieties differ in the quantity of phenolic acids present in the berries (Rodrigues *et al*., 2001; Morel-Salmi *et al*., 2006; Morata *et al*., 2007; Rentzsch *et al*., 2007). The presence of the three hydroxycinnamic acids (caffeic acid, ferulic acid and *ρ*-coumaric acid) in grapes originate from their bound form with tartaric acid known as caftaric acid, fertaric acid and coutaric acid, respectively. General concentrations for hydroxycinnamic acids present in *Vitis vinifera* juice (oxidative and hydrolytic losses prevented) are about 150 mg/L of caftaric acid, 20 mg/L coutaric acid and 1 mg/L fertaric acid (Boulton *et al*., 1996). Other varieties have been identified to have higher amounts of hydroxycinnamic acids for example Grenache wines can contain between 270–460 mg/L caftaric acid (Morel-Salmi *et al*., 2006). Other wine varieties mean values for caftaric acid range from 50–60 mg/L (Rentzsch *et al*., 2007).

Other factors that can influence the amount of hydroxycinnamic acids found in grapes include the origin of the grapes, degree of ripeness, exposure to sunlight, viticultural practises and the disease status of the grapes (Boulton *et al*., 1996; Van Wyk & Rogers, 2000; Geny *et al*., 2003). These compounds increase as the ripening process of berries progress (Boulton *et al*., 1996). Van Wyk and Rogers (2000) proposed that lower ferulic acid concentrations in grapes, and therefore a reduction in 4-vinylguaiacol, could be obtained through protecting grape clusters from sunlight exposure with proper canopy management together with early harvesting. It has been found that during the course of disease development of *Botrytis cinerea* the levels of specifically the different hydroxycinnamic acids can change significantly (Geny *et al*., 2003).

Dias *et al.* (2003b) studied the conversion of 4-vinylphenols into 4-ethylphenols by *D. bruxellensis* in synthetic media. They found that 4-vinylphenols could be used as a precursor for 4-ethylphenol production in the absence of *ρ*-coumaric acid. It was also shown that if they added 4-vinylphenols to the growth media, the production of 4-ethylphenols proceeded in a similar way as when glucose was used as the only carbon and energy source (**Figure 2.4**).

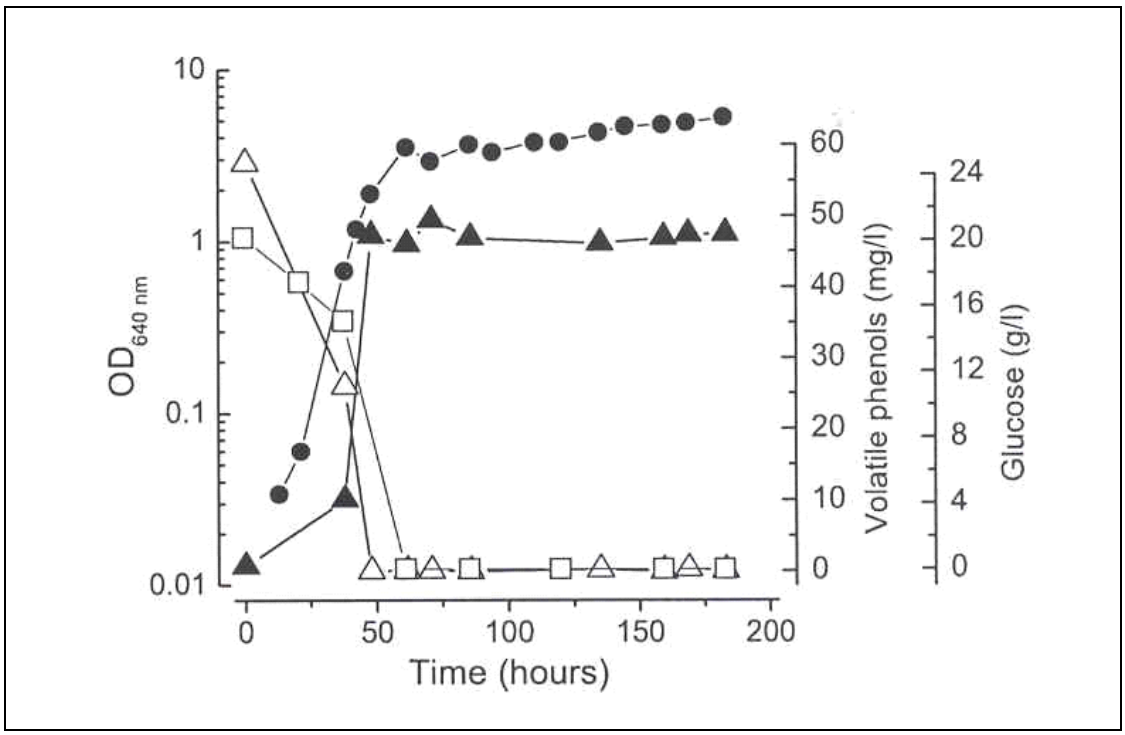

FIGURE 2.4
The growth of *B. bruxellensis* ISA 1791 in YNB with amino acids added in the presence of 75 mg/L 4-vinylphenol and 20 g/L glucose. Symbols: ●, optical density; ▲, 4-ethylphenol; ∆, 4-vinylphenol; □, glucose (Dias *et al*., 2003b).

2.4.3.3 Species and strains of *Brettanomyces* yeast

The taxonomy of *Brettanomyces* and *Dekkera* spp. has changed significantly during the last few years. The use of DNA-based methods replaced morphological and physiological classification methods and a number of physiologically diverse species, for example *Dekkera intermedia* and *Brettanomyces lambicus*, has been reclassified as *Brettanomyces/Dekkera bruxellensis* (Smith *et al*., 1990). Currently the genus *Brettanomyces* and its sexual anamorph *Dekkera* are divided into five species including *B. bruxellensis, B. anomalus, B. custersianus, B. naardenensis* and *B. nanus* (Egli & Henick-Kling, 2001). To date, *B. bruxellensis* and more recently *B. anomalus* have been associated with wine. However, *B*. *bruxellensis* is considered to be the dominant species responsible for the production of phenolic off-flavours in wines (Cocolin *et al.,* 2004).

Different species of *Brettanomyces* yeast vary in their ability to produce volatile phenols. It has previously been reported that *B. intermedius* (reclassified to *B. bruxellensis*) and *B. anomalus* can decarboxylate hydroxycinnamic acids to the vinyl derivatives, which can then be reduced to the ethyl derivatives (Heresztyn 1986b; Chatonnet *et al*., 1992). Heresztyn (1986b) investigated the metabolism of hydroxycinnamic acids and found that *B. intermedius* could metabolise ferulic acid, *ρ*-coumaric acid and sinapic acid, but not caffeic acid, vanillic acid or syringic acid. The same authors also showed that *B. anomalus* could metabolise caffeic acid but not sinapic acid. It is therefore suggested that differences in the specificity of the cinnamate

decarboxylase enzyme exist between species and therefore the ability of the species to metabolism hydroxycinnamic acids will vary (Edlin *et al*., 1995).

In a study by Fugelsang and Zoecklein (2003) they investigated the population dynamics and effects of different strains of *B. bruxellensis* on Pinot noir wines. The different strains showed a significant variation in the growth rate and the population density during the stationary phase. Differences among the strains were also detected during sensory evaluation of the wines. In another study *B. bruxellensis* isolates also varied towards the impact they had on the phenolic profile of a wine. This suggests that it is important to be able to detect the occurrence of specific strains in wine and to predict their possible spoilage capacity (Silva *et al*., 2005; Curtin *et al*., 2007).

Conterno *et al*. (2006) found high variability in the production of volatile phenols amongst different strains of *B. bruxellensis* isolated from wines. A selection of the isolates produced high and others lower amounts of 4-ethylphenol and 4-ethylguaiacol. Valentão *et al*. (2007) investigated the production of volatile phenols of nine strains of *B. bruxellensis* in Dão red wines. They found that the presence of certain strains led to the deterioration of red wines, because of the production of very high levels of 4-ethylphenol.

2.4.3.4 Oxygen

As previously mentioned *Brettanomyces* species display a negative Pasteur effect called the Custer's effect, where oxygen stimulates their fermentation ability (Boulton *et al*., 1996). Ciani and Ferraro (1997) evaluated the influence of oxygen levels on the production of acetic acid by *Brettanomyces* yeast during the winemaking process and their results indicated that different oxygen levels had a strong influence on the metabolic activity of this yeast. It was found that alcoholic fermentation by only *Brettanomyces* yeast under full aerobiosis led to the production of high concentrations of acetic acid causing rapid cell death and eventually stuck fermentations. It was shown that semi-aerobiosis was the most favourable conditions for *Brettanomyces* spp. to ferment and this led to higher ethanol concentrations and lower levels of acetic acid. During anaerobic conditions fermentation rates were slower due to reduced growth of these yeasts and a limited amount of acetic acid was produced.

In support of this, Aguilar Uscanga *et al*. (2003) showed that the growth of *Brettanomyces* spp. was stimulated by moderate aeration. They found that the higher the oxygen supply, the greater the acetic acid production and the lower the ethanol production. Abbott and Ingledew (2005) investigated the importance of aeration strategy in whole corn mash fermentations contaminated by *Brettanomyces* spp. It was found that aeration during fermentation lead to lower ethanol production. They concluded that the aeration strategy is a key parameter with regards to the negative effect exhibited in fuel alcoholic fermentations contaminated by *Brettanomyces* spp.

A study by Du Toit *et al*. (2005) focussed on the effects of different oxygen concentrations on the viability and culturability of a *B. bruxellensis* strain. It was clear that addition of oxygen to wine containing low concentrations of sulphur dioxide (lower

that 25 mg/L free SO_2) could sustain the growth and survival of *Brettanomyces* spp. They suggested that if a wine is contaminated with *Brettanomyces* spp. exposure to excessive amounts of oxygen should be avoided. It was also suggested that due to a lack of substantial information, further research should be conducted to determine the effect of oxygen on the growth of different strains of *Brettanomyces* yeast in wines from different regions and grape varieties.

Micro-oxygenation of wine is becoming a very popular technique to enhance colour stability and intensity, softening of tannins and to decrease reductive and vegetative aromas. The process of micro-oxygenation involves the introduction of controlled amounts of oxygen into wines (Parish *et al*., 2000). A study by Du Toit *et al*. (2006) investigated the effect of micro-oxygenation on wine spoilage microorganisms of different South African red wines. It was found that prolonged micro-oxygenation in older red wines led to an increase in the barnyard and medicinal character as a result of an increase in *Brettanomyces* yeast populations. It was suggested that the sulphur dioxide levels of wines should be monitored during micro-oxygenation to limit the growth of *Brettanomyces* yeast and the production of medicinal off-flavours.

2.4.3.5 Sulphur dioxide

It has been shown that the only way to limit the growth of *Brettanomyces* yeast in wines is to maintain a sufficient concentration of molecular sulphur dioxide (SO_2) during the aging process (Licker *et al*., 1998). Du Toit *et al*. (2005) investigated the inhibitory effect of SO_2 on the viability and culturability of *B. bruxellensis* in wine. They found that the growth of a *B. bruxellensis* strain was inhibited in YPD media containing 0.25 mg/L molecular SO_2 and that it also drastically reduced their viability. They also noticed that some cells had higher epifluorescence counts than others after being exposed to molecular SO_2. A possible explanation could be that some cells are more resistant to SO_2 or that some are able to take up more SO_2 than others. To avoid *Brettanomyces* contamination it is very important to monitor the molecular SO_2 levels of wines frequently and adjust it to 25-30 mg/L free SO_2. It is important to note that the molecular SO_2 concentration in wines is pH dependent and that at a lower pH a higher percentage of free SO_2 is present in the molecular form. Lower pH values should therefore be maintained to ensure the effective antimicrobial action of SO_2 (Du Toit *et al*., 2005; Suárez *et al*., 2007).

2.4.3.6 Temperature

Techniques used during winemaking to improve the extraction of phenolic compounds can indirectly lead to the increased production of volatile phenols in *Brettanomyces* yeast infected wines. These techniques include the thermovinification, maceration heating and elevated temperatures during tank and barrel storage of wine. Heating at the end of maceration leads to an increase in the extraction of phenolic acids and therefore an increase in the production of volatile phenols by *Brettanomyces* yeast (Gerbaux *et al*., 2002).

It has been observed that wines are more susceptible to the development of phenolic off-flavours during warmer months. At higher temperatures the production rate of 4-ethylphenol accelerate possibly as a result of more favourable growth of *Brettanomyces* yeasts. It is therefore suggested that lower temperatures should be maintained in cellars to delay the process of 4-ethylphenol formation (Dias *et al*., 2003b).

Couto *et al*. (2005) investigated the heat resistance of three *Brettanomyces* strains at different temperatures ranging from 32.5 to 55°C in tartrate buffer solution (pH 4) and in wines. They observed that in the buffer solution thermal inactivation only began at temperatures above 50°C. It was shown that heat sensitivity of the *Brettanomyces* strains was much higher in wine than in the buffer solution. Significant inactivation of *Brettanomyces* yeasts was obtained when temperatures of wine reached 35°C.

2.4.3.7 Ethanol concentration

The toxicity of ethanol on yeasts is associated with the solubility in their membrane lipids. Cell walls of anaerobic yeasts have deficiencies in sterols, phospholipids and polyunsaturated fatty acids reducing their resistance to ethanol (Larue *et al*., 1980; Medawar *et al*., 2003).

It has been stated by various authors that *Brettanomyces* yeasts is sensitive to high ethanol levels and that wines with high levels of ethanol did not show high levels of 4-ethylphenol (Rodrigues *et al*., 2001; Dias *et al*., 2003b). Dias *et al*. (2003b) studied the inhibitory effect of ethanol on *Brettanomyces* yeast growth and 4-ethylphenol production at 4%, 6%, 8%, 10%, 12% and 13%(v/v). The increased ethanol concentration [up to 10% (v/v)] did lower the growth rates of *B. bruxellensis* but did not have an effect on the maximum production of 4-ethylphenol. At 12% (v/v) the growth and 4-ethylphenol production of *Brettanomyces* spp. was significantly lower and a total inhibition occurred at 13% (v/v).

Medawar *et al*. (2003) investigated the relationship between the lag phase duration of *B. intermedius* and the ethanol concentration in an alcoholic medium. Their results showed an increase of the lag phase duration, a reduction in the specific growth rate and biomass yield with an increase in ethanol concentration and no growth was detected at ethanol concentrations of 91 g/L. This suggests a critical ethanol concentration of 91 g/L for *B. intermedius* and above this concentration their cells cease to reproduce.

An investigation by Silva *et al*. (2004) determined the capacity of *Brettanomyces* spp. to grow and survive in wine with high ethanol levels [9 to 12 % (v/v)] and low glucose concentrations. They found that the growth of *B. bruxellensis* showed more resistance to the toxicity of ethanol compared to *S. cerevisiae.* This could mean that dry wines with high alcohol concentrations can possibly host *Brettanomyces* yeasts.

2.4.3.8 Yeast lees

The maturation of wine in contact with yeast lees is a technique used frequently in the wine industry. During aging of wine on lees, yeast autolysis releases small molecular weight materials into the wine for example amino acids, vitamins, mannoproteins and other nitrogenous compounds (Boulton *et al*., 1996). There are many advantages in the release of cell wall macromolecules into wine including encouraged growth of malolactic bacteria during MLF, contribution to aroma and body of wines and a decrease in astringency and bitterness of tannins (Fleuillat, 2003).

Previous studies have shown that *Brettanomyces* spp. grew easily in synthetic media containing autolysed *S. cerevisiae* cells, but that the concentration of ethylphenols was lower compared to media without autolysed *S. cerevisiae* cells (Guilloux-Benatier *et al*., 2001). The reduction of ethylphenols found in wines aged with lees can possibly be explained by the adsorption of phenolic compounds by yeast cell wall fragments (Morata *et al*., 2003, 2005; Suárez *et al*., 2007). It was also observed that yeast lees are able to adsorb anthocyanins and therefore influence the colour stability of red wines (Morata *et al*., 2003).

The ability of *S. cerevisiae* yeast lees to adsorb volatile phenols has been investigated and it was found that 4-ethylphenol and 4-ethylguaiacol levels decreased in red wines containing yeast lees compared to wines aged without lees (Guilloux-Benatier *et al*., 2001; Chassagne *et al*., 2005). It was found that the adsorption of hydrophobic solutes, such as 4-ethylphenol and 4-ethylguaiacol by yeast cells occurs mainly by surface binding. A possible limiting factor for the ability of cells to adsorb volatile phenols could be the availability of binding sites on the adsorbing cells (Chassagne *et al*., 2005). Other factors that can influence the uptake of volatile phenols by *S. cerevisiae* yeast include temperature, pH, ethanol, autolytic state of the yeast cells and the interaction between wine constituents and the yeast cells (Lubbers *et al*., 1994; Ramirez-Ramirez *et al*., 2004; Chassagne *et al*., 2005). It was found that with an increase in temperature, pH and ethanol the amount of volatile phenols adsorbed, decreases (Chassagne *et al*., 2005). The reduced adsorption of volatile phenols with an increase in temperature and pH can possibly be explained by the occurrence of polar bindings in addition to hydrophobic interactions between volatile phenols and yeast cell surfaces.

2.4.3.9 Enzymes used during wine production

It is a very common practise to use certain commercially available enzymes during the production of wine. This allows the better extraction of phenolic compounds including colour extraction, improve clarification, improve yield and improve the sensory quality of wines (Dugelay *et al*., 1993; Lao *et al*., 1997). The enzymatic activity of *Aspergillus niger* and *Trichoderma harzianum* fungi are used in the commercial preparations of pectinase and β-1,3-1,6-glucanase, but these preparations include secondary activities that can potentially affect the quality of wines. This includes the release of free phenolic acids from their tartaric bound form through the activity of cinnamoyl-esterases.

Brettanomyces yeast can consequently transform free available phenolic acids resulting in an increase in the production of volatile phenols (Gerbaux *et al*., 2002; Lao *et al*., 1997). In a study by Dugelay *et al*. (1993) it was shown that higher levels of vinylphenols occurred in wines made from juices initially treated with certain pectinase enzyme preparations. They found significant cinnamoyl esterase activity in five out of the eight enzyme preparations tested and suggested that consideration should be taken when choosing enzyme preparations for wine production to avoid the presence of undesirable enzymatic activity that could contribute to the formation of phenolic off-flavours.

2.4.4 OTHER DEFAULTS CAUSED BY *BRETTANOMYCES*

In addition to volatile phenols, *Brettanomyces* yeast is also known to produce high levels of acetic acid in wines (Boulton *et al*., 1996; Ciani & Ferraro 1997). The presence of high levels of acetic acid is undesirable, because of the negative sensory attributes associated with an increase in the volatile acidity of wines (Boulton *et al*., 1996; Arvik & Henick-Kling, 2002).

Brettanomyces spp. have also been found to be responsible for the formation of 2-acetyl-1,4,5,6-tetrahydropyridine and 2-acetyl-3,4,5,6-tetrahydropyridine in the presence of lysine and ethanol (Heresztyn *et al*., 1986a). Tetrahydropyridines produce a mousiness described as an extremely unpleasant odour in wines reminiscent of the smell of mouse urine or acetamide (Heresztyn *et al*., 1986a; Boulton *et al*., 1996; Licker *et al*., 1998; Snowdon *et al*., 2006). A study by Grbin and Henschke (2000) demonstrated the ability of *Brettanomyces* yeast to produce mousy off-flavours in grape juice and wine. The occurrence of mousiness in wines does not arise from *Brettanomyces* alone. *Lactobacillus* spp. can also synthesize tetrahydropyridines (Heresztyn *et al*., 1986a). Wines with this off-taint are considered to be unpalatable and there is no sufficient method in place to remove these compounds (Grbin & Henschke, 2000). It is therefore very important to control the growth of wine microorganisms with the ability to produce these compounds.

The production of medium-chain fatty acids by *B. bruxellensis*, for example iso-valeric acid, in wine can also cause off-flavours. Wine with low concentrations of this compound can be described as having a rancid, cheesy, sweaty and putrid aroma (Miranda-López *et al*., 1992; Renouf & Lonvaud-Funel, 2007).

Other defects caused by *Brettanomyces*/*Dekkera* yeasts include the formation of sediment or haziness in wines (van der Walt & van Kerken, 1958). It has also been reported that *Brettanomyces* yeast contamination can have an impact on the colour of red wine (Mansfield *et al*., 2002). The compound responsible for the red pigment in *Vitis vinifera* grapes is mono-glucosylated anthocyanins. *Brettanomyces bruxellensis* yeast strains exhibit β-D-glucosidase activity with the ability of hydrolysing glucose and this leads to the formation of anthocyanidin aglycone that results in colour loss and instability (Huang, 1955; Boulton *et al*., 1996; Mansfield *et al*., 2002).

2.4.5 CONTROLLING *BRETTANOMYCES* YEASTS AND HIGH LEVELS OF VOLATILE PHENOLS IN WINES

To prevent the contamination of winery and winery equipment by *Brettanomyces* yeast is the first step in controlling the contamination of wines and the production of phenolic off-flavours. Identifying the conditions and possible factors that could stimulate or inhibit the growth and metabolism of *Brettanomyces* yeast is very important in effective control of this spoilage microorganism (Boulton *et al*., 1996; Arvik & Henick-Kling, 2002).

The control of *Brettanomyces* yeast contamination could already start in the vineyard. Although this yeast does not seem to be present in high numbers on grapes, damaged grapes may contain higher microbial loads than healthy grapes. Therefore the damaging of grapes during harvest should be avoided and SO_2 can be added as soon as the grapes arrive at the winery (Arvik & Henick-Kling, 2002; Renouf & Lonvaud-Funel, 2007; Suárez *et al.,* 2007). Renouf and Lonvaud-Funel (2007) indicated that the grape berry could be the primary source of *B. bruxellensis* contamination in wine. It is therefore very important for grape growers and winemakers to control this spoilage yeast from an early stage during the production of wine and if *Brettanomyces* yeasts has been detected it is best to avoid the use of damaged or low quality grapes and good SO_2 practises should be implemented as soon as possible.

Winemaking techniques that enhance the extraction of phenolic compounds from grapes could indirectly lead to the production of higher volatile phenol levels in wines contaminated with *Brettanomyces* yeast. When choosing commercial enzyme preparations care should be taken to avoid preparations with high cinnamoyl-esterase activity that could lead to elevated levels of free hydroxycinnamic acids and therefore higher potential volatile phenol production. Long maceration periods at elevated temperatures should also be avoided (Gerbaux *et al*., 2002). Maintaining lower temperatures during aging of wines can also induce lower levels of volatile phenols (Dias *et al*., 2003b).

The judicious use of SO_2 during the production of wine could reduce the growth of unwanted microbes including, *Brettanomyces* yeasts (Boulton *et al*., 1996; Suárez *et al*., 2007). Maintaining stable concentrations of SO_2 is difficult, but the critical additions should occur at picking or crushing and after MLF before barrel aging, as these are the most critical stages that require antimicrobial protection (Suárez *et al*., 2007; Oelofse *et al*., 2008). As previously mentioned SO_2 is more effective at lower pH values and therefore it is important to note that the potential occurrence of undesirable microorganisms is higher in higher pH wines found typically in warmer winegrowing countries (Du Toit *et al*., 2005; Oelofse *et al*., 2008). Maintaining pH levels of below 3.6 is a good winemaking practise to curtail spoilage by various microorganisms. It has been suggested that the use of between 0.5 and 0.8 mg/L molecular SO_2 is recommended minimise *Brettanomyces* yeast contamination (Suárez *et al*., 2007). It is also important to note that the sensitivity of *Brettanomyces* yeast to the antimicrobial action of SO_2 is strain dependent and genetic variability between strains exists (Godden *et al*., 2004).

The use of dimethyl dicarbonate (DMDC), commercially known as Velcorin®, has been recommended as an alternative inhibitor for *Brettanomyces* yeast. Recent studies showed that although other fermentative yeasts are inhibited by 250-400 mg/L DMDC, a dosage of 400 mg/L could not completely inhibit the growth of *B. anomalus* (Delfini *et al*., 2002).

It has been reported that chitosan, a polysaccharide derived from chitin, has a selective action on *Brettanomyces* yeast. This compound causes a delay in the log phase of *Brettanomyces* spp. in mixed cultures with *S. cerevisiae*. The growth of *B. bruxellensis* and *B. intermedius* was inhibited in the presence of 3-6 g/L chitosan. These concentrations did not affect the development of *S. cerevisiae* (Góme-Rivas *et al*., 2004). Kiskó *et al*. (2005) evaluated the possibility to use chitosan as a natural food preservative and found that some spoilage yeasts were inactivated at a concentration of 0.05-0.1% at 25°C.

Other additives that could be used as inhibitory substances against *Brettanomyces* yeasts include weak acids such as sorbic, benzoic and fumaric acids (Suárez *et al*., 2007). However, the use of these substances is not authorised in the production of wine in certain countries. The use of ascorbic acid as antioxidant can reduce the presence of oxygen and therefore restrict the growth of *Brettanomyces* yeast (Suárez *et al*., 2007).

As previously mentioned, the growth of *Brettanomyces* yeast is stimulated by the presence of oxygen (Aguilar Uscanga *et al*., 2003; Du Toit *et al*., 2005). Therefore by avoiding unnecessary exposure of wines to oxygen winemakers can restrict the growth of *Brettanomyces* yeast. If wines are contaminated with *Brettanomyces* it is suggested that the micro-oxygenation technique should not be implemented (Suárez *et al*., 2007).

Although it might be suggested that the presence of yeast lees can lead to proliferation of *Brettanomyces* yeast growth, it has been shown that yeast lees has the ability to adsorb volatile phenols resulting in lower organoleptic defects in wines (Chassagne *et al*., 2005). It has to be considered that yeast lees is very rich in nutrients and could encourage the growth of potential spoilage microorganisms. Therefore other factors including avoiding excessive exposure to oxygen and maintaining high levels of SO_2 should accompany the use of this technique to lower volatile phenol levels in wines (Oelofse *et al*., 2008). Delayed racking from the lees can also lead to increased levels of 4-ethylphenol in wines as the low levels of SO_2 found during this stage provides more favourable conditions for *Brettanomyces* yeast proliferation (Chatonnet *et al*., 1995).

Other techniques suggested to reduce *Brettanomyces* populations include filtration (0.45 µm membranes) or cross flow filtration (0.22 µm membranes) and the use of protein clarification products (gelatine, egg white, potassium caseinate and casein). The disadvantages associated with these techniques include the loss of colour and wine aroma (Murat & Dumeau, 2003; Oelofse *et al*., 2008). Using reverse osmosis can also significantly reduce the concentration of 4-ethylphenol and 4-ethylguaiacol in red wines, but this is accompanied by a loss of other favourable aroma compounds (Ugarte *et al*., 2005).

A possible biological control for *Dekkera*/*Brettanomyces* includes the fungicidal effect of two killer toxins produced by *Pichia anomala* (DBVPG 3003) and *Kluyveromyces wickerhamii* (DBVPG 6077), called Pikt and Kwkt, respectively. These toxins show a fungicidal effect against *D. bruxellensis* for at least 10 days and show potential for future use as antimicrobial agents against *Dekkera*/*Brettanomyces* during wine aging and storage (Comitini *et al*., 2004).

Maintaining good cellar hygiene and sanitation practises, such as frequent sterilisation of winery equipment, walls and floors, is pivotal in effective control of *Brettanomyces* yeast contamination (Oelofse & Du Toit, 2006b).

2.4.6 THE INTERACTION BETWEEN *BRETTANOMYCES* AND OTHER WINE MICROORGANISMS

The production of wine involves the complex interaction between yeast and bacteria in grape must and wine. Yeast populations associated with wine can be divided into non-*Saccharomyces* and *Saccharomyces* yeasts. During fermentation a complex mixture of yeast strains proliferate and expire according to their adaptation to the fermenting must (Renouf *et al*., 2006).

Dias *et al*. (2003b) studied the growth and production of 4-ethylphenol in mixed cultures in synthetic media and grape juice. The main significance of their results for the growth of *S. cerevisiae* ISA 1000 and *D. bruxellensis* ISA 1791 in synthetic media are shown in **Figure 2.5**. The cell numbers of *S. cerevisiae* increased to a maximum of 3 x 10^9 cfu/mL and then began to lose viability at the end of fermentation. The population of *D. bruxellensis* also increased from the initial cell numbers of 10^4 cfu/mL to 5 x 10^9 cfu/mL. At the beginning of fermentation the amount of 4-vinylphenol increased and the conversion to 4-ethylphenol started when *D. bruxellensis* growth reached the stationary phase. Results in both synthetic media and grape juice indicated that 4-vinylphenol was produced during alcoholic fermentation by *S. cerevisiae* and that *D. bruxellensis* populations survived during this time to produce 4-ethylphenol in the post-fermentation period. The production of 4-vinylphenols by *S. cerevisiae* can therefore influence the final concentration of 4-ethylphenol produced by *D. bruxellensis*.

Renouf *et al.* (2006) conducted an investigation into the interactions between yeasts during the initial stages of wine production. It was found that *B. bruxellensis* is better adapted than other non-*Saccharomyces* yeasts to grow in grape must and during the process of alcoholic fermentation. *Brettanomyces bruxellensis* also appears to be more resistant to increased ethanol during the fermentation process compared to *S. cerevisiae,* and this could explain why *B. bruxellensis* yeast was found to be the dominant yeast species at the end of alcoholic fermentation. The beginning of MLF is therefore considered to be the critical stage during wine production where *B. bruxellensis* contamination might take place.

There has only been a limited amount of research done on the interaction between *Brettanomyces*/*Dekkera* yeast and LAB during MLF. In a recent study by Romano *et al*. (2008) they investigated the growth and volatile phenol production of *B. bruxellensis* in

wine before the completion of alcoholic fermentation (stuck fermentation). These results were also compared with the results found when contamination occurred after MLF. It was found that *Brettanomyces* yeast had a shorter lag phase, grew faster, obtaining higher maximum biomass levels and 4-ethylphenol development when contamination occurred during stuck alcoholic fermentation. They suggested that bacterial activity during MLF could increase the toxicity of the wine towards the growth of *B. bruxellensis*. This suggests that wines with higher residual sugars could possibly develop more of the phenolic off-taint than dry wines.

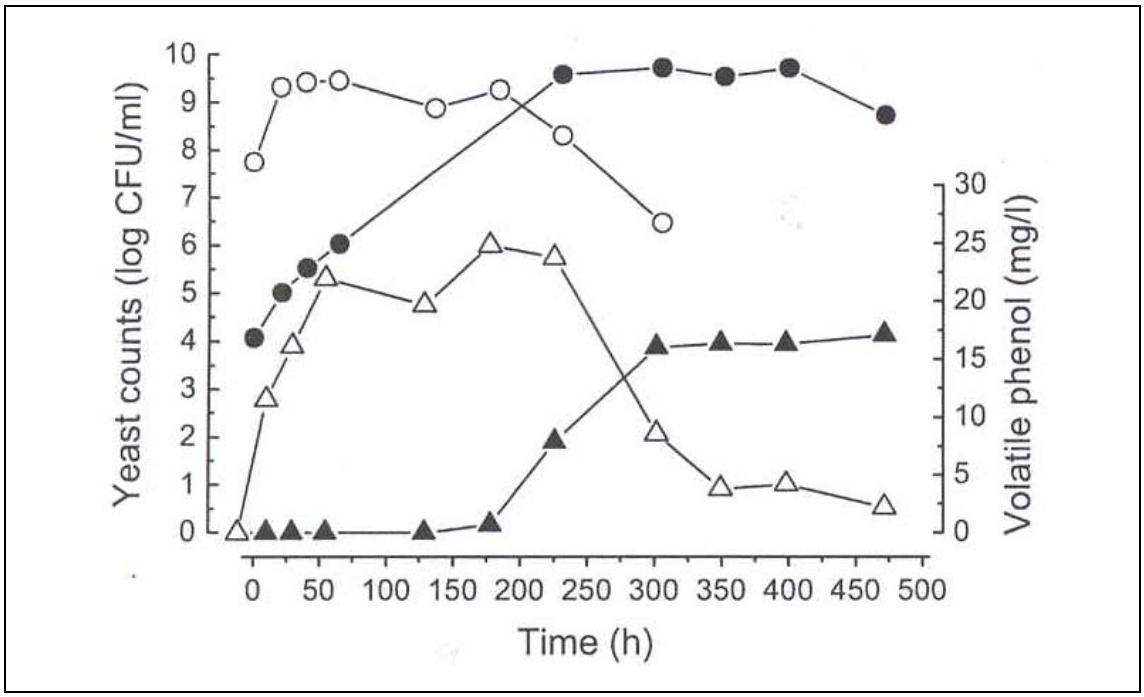

FIGURE 2.5
The growth and volatile phenol production of mixed cultures of *S. cerevisiae* ISA 1000 and *D. bruxellensis* ISA 1791 grown in YNB supplemented with 100g/L *ρ*-coumaric acid, 100g/L glucose and 100g/L fructose. Symbols: ○, colony counts of *S. cerevisiae*; ●, colony counts of *D. bruxellensis;* Δ, 4-vinylphenol; ▲, 4-ethylphenol (Dias *et al*., 2003b).

2.5 THE PRODUCTION OF VOLATILE PHENOLS BY *SACCHAROMYCES CEREVISIAE*

For the successful onset and completion of alcoholic fermentation, grape juice is typically inoculated with yeast starter cultures. *Saccharomyces cerevisiae* and other related *Saccharomyces* species are usually selected for the use in starter cultures, because of their positive contribution to wine production including high fermentation rates, successful completion of fermentation, good ethanol, temperature, SO_2 and sugar tolerance, and favourable aroma production (Boulton *et al*., 1996).

As previously mentioned 4-vinylphenol and 4-vinylguaiacol are compounds found naturally in wines and play an important role in the aroma characteristics of wine. Chatonnet *et al*. (1993) stated that only white wines contain significant amounts of the vinylphenols and that above a certain concentration can be responsible for phenolic or pharmaceutical aromas in wine. They also studied the synthesis of volatile phenols by

S. cerevisiae during the production of wine. It was found that *S. cerevisiae* was only able to convert the hydroxycinnamic acids, *ρ*-coumaric acid and ferulic acids to 4-vinylphenol and 4-vinylguaiacol, respectively, and could not produce the ethyl derivatives. Enzymatic conversion was due to the decarboxylase activity of these yeasts. The decarboxylation of the cinnamic acids is stereo specific and it only occurs with the trans (*E*) isomer (Chatonnet *et al*., 1993). It has been shown that the cinnamate decarboxylase enzyme of *S. cerevisiae* is strictly intracellular with an optimum pH of about 6.5 (Chatonnet *et al*., 1993). This enzyme is only active during alcoholic fermentation and no vinyl derivatives can be synthesised in dry wines by *S. cerevisiae* yeasts.

During alcoholic fermentation of grape must different strains of *S. cerevisiae* produced different levels of volatile phenols (**Figure 2.6**). *S. cerevisiae* strains therefore vary in their ability to decarboxylate free hydroxycinnamic acids. Phenolic off-flavour (POF) negative strains can therefore be selected to minimize the possible production of vinylphenol (Chatonnet *et al*., 1993). Van Wyk and Rogers (2000) also investigated the ability of different *S. cerevisiae* wine yeast strains to decarboxylate ferulic acid to 4-vinylguaiacol in Kerner wines. It was found that commercial yeast strains could be divided into high and low producers of 4-vinylguaiacol. It is therefore suggested that low 4-vinylguaiacol producing commercial *S. cerevisiae* yeast strains should be selected for the fermentation of grape varieties with high levels of ferulic acid precursors. This study was only done in one grape varietal and further research is necessary to confirm these results in other grape varieties.

Shinohara *et al*. (2000) examined the phenolic off-flavour production of *Saccharomyces* and non-*Saccharomyces* yeasts (see also section 2.6). Their results showed frequent distribution of phenolic yeasts in the winemaking environment. Segregation of the phenolic off-flavour phenotype and Southern blot analysis of the phenolic wine yeasts (*S. cerevisiae*) suggested that the phenolic off-flavour production is controlled by a phenyl acrylic acid decarboxylase gene (PCR product of the 869 bp fragment containing the *PAD1* or *POF1* gene) (Shinohara *et al*., 2000). The *PAD1* gene encodes phenyl acrylic acid decarboxylase and is a single copy gene in the yeast genome (Clausen *et al*., 1994). Shinohara *et al*. (2000) confirmed the results of Clausen *et al*. (1994) and that the *PAD1* gene is only located on chromosome IV of *Saccharomyces* yeast.

The *PAD1* gene of *S. cerevisiae* is responsible for encoding a protein (Pad1p) that has a low activity against ferulic and *ρ*-coumaric acids. It was therefore suggested that this activity is not sufficient to have an effect on the aroma of wine. This provoked an experiment to optimise the decarboxylase activity on phenolic acids by over-expressing a *Bacillus subtilis* phenolic acid decarboxylase gene (*padC*), the *Lactobacillus plantarum ρ*-coumaric acid decarboxylase gene (*pdc*) and the *S. cerevisiae* phenyl acrylic acid decarboxylase gene (*PAD1*) in a laboratory *S. cerevisiae* strain (Smit *et al*., 2003). The overexpression of the *padC* and *pdc* genes resulted in higher enzymatic activity, but the over-expressed *PAD1* gene did not significantly improve the

functionality of the Pad1p enzyme. The *padC* and *pdc* genes were also over-expressed in a commercial VIN 13 yeast stain. During small-scale fermentations with grape juice the laboratory and commercial yeast with the *padC* and *pdc* genes showed increase in volatile phenol production, because of higher enzymatic activity (Smit *et al*., 2003). This work could lead to possible ways in which to control the concentration of volatile phenols in wine (Swiegers *et al*., 2005).

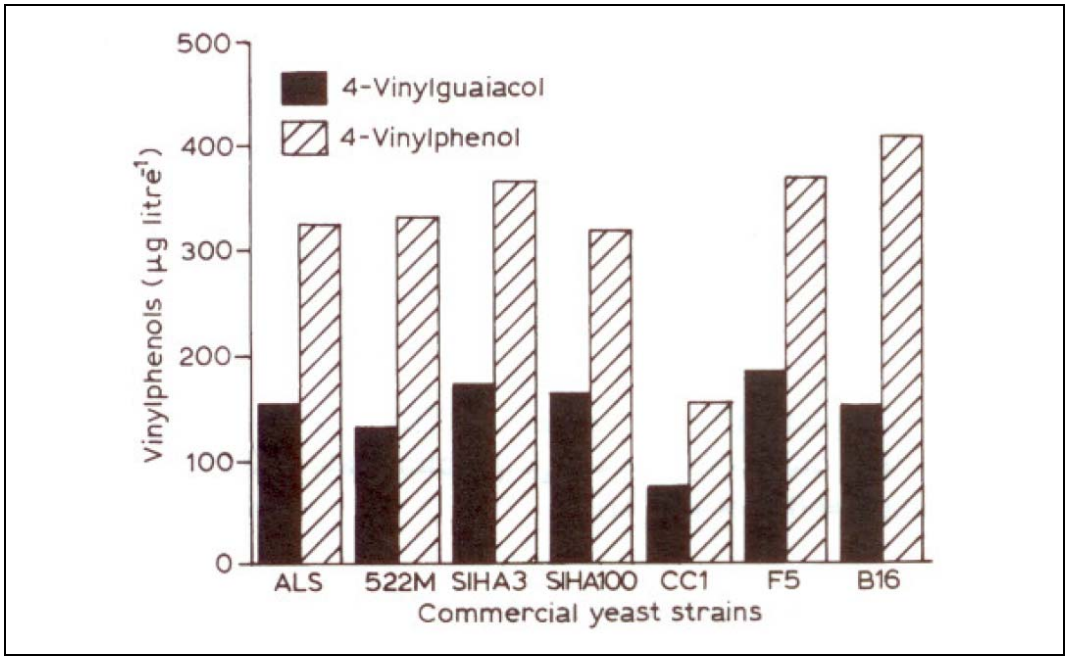

FIGURE 2.6
Influence of the *S. cerevisiae* yeast strain on the vinylphenol concentration of a white wine (Sauvignon blanc variety) (Chatonnet *et al.,* 1993).

2.6 THE PRODUCTION OF VOLATILE PHENOLS BY OTHER INDIGENOUS YEAST POPULATIONS

Although *S. cerevisiae* is responsible for alcoholic fermentation of grape juice, other non-*Saccharomyces* yeasts are always present in varying numbers during fermentation. The growth of these yeasts can contribute positively or negatively to the final aroma characteristics of the wine (Egli *et al*., 1998).

Investigations on the production of phenolic off-flavours in a grape juice media supplemented with ferulic and ρ-coumaric acid, found that strains of *Rhodotorula, Candida, Cryptococcus, Pichia, Hansenula* and *Brettanomyces* produced high levels of phenolic off-flavours (Shinohara *et al*., 2000). It was speculated that the orthologs of the *PAD1* gene is present in these yeast species that encodes a similar decarboxylase that are responsible for the conversion of ferulic acid and *ρ*-coumaric acid into 4-vinylguaiacol and 4-vinylphenol, respectively (Shinohara *et al*., 2000). This is the first enzyme in the two-step path for the formation of 4-ethylguaiacol and 4-ethylphenol responsible for the so-called "*Brettanomyces* character" of a wine (Dias *et al*., 2003a).

Furthermore Dias *et al*. (2003a) have isolated *Pichia guilliermondi* strains from grapes, grape juice and from winery equipment that have been in contact with grape

juice, but not with wines. They showed that some strains of had high conversion rates of *ρ*-coumaric acid to 4-ethylphenol in synthetic media. Other yeast strains including *Candida cantarelli, Candida wickerhamii, Debaryomyces hansenii, Kluyveromyces lactis* and other strains of *P. guilliermondii* were found to be low producers of 4-ethylphenol in synthetic media. This indicates the possibility of these yeasts to produce 4-ethylphenol during the winemaking process. In a later study by Martorell *et al*. (2006) their results corresponded to that of Dias *et al*. (2003a) that some strains of *P. guilliermondii* could produce high levels of 4-ethylphenol in synthetic media. They were able to identify the presence of *P. guilliermondii* on grapes, stems, winery equipment during harvest, from fruit flies and in wines and oak barrels. It was suggested that the ability to produce 4-ethylphenol in wine should be evaluated to assess the potential risk of the formation of spoilage products by these yeast strains (Martorell *et al*., 2006).

In an ongoing study by Barata *et al*. (2006) the ability of *P. guilliermondii* to produce 4-ethylphenol in wines was investigated. For this study they selected *P. guilliermondii* strains that showed high conversion rates of *ρ*-coumaric acid to 4-ethylphenol in synthetic media to assess the same ability in real wine. These yeasts were not able to survive and produce 4-ethylphenol in red wines with average ethanol concentrations ranging from 10 to 12% (v/v) together with a pH of 3.5. If *P. guilliermondii* was grown in a single culture in grape juice, the selected strain was able to produce high levels of 4-ethylphenol before the onset of fermentation by *S. cerevisiae*. The co-inoculation of *S. cerevisiae* and *P. guilliermondii* into grape juice showed that *P. guilliermondii* is sensitive to the dominating growth effect of *S. cerevisiae* and did not lead to the production of 4-ethylphenol. High levels of 4-ethylphenol were obtained when *P. guilliermondii* was inoculated 72 hours before inoculation together with the inoculation of low cell densities (10^2 cfu/mL) of *S. cerevisiae*. The study concluded that the possible production of high levels of 4-ethylphenols by *P. guilliermondii* could occur in contaminated grape juice if the inoculation of the *S. cerevisiae* starter culture is delayed for example if pre-fermentative maceration techniques are used (Barata *et al*., 2006). The possibility that other strains of *P. guilliermondii* and wine microbes could produce high levels of ethylphenols in wine should not be excluded.

2.7 THE PRODUCTION OF VOLATILE PHENOLS BY LACTIC ACID BACTERIA

LAB are responsible for MLF relating to the conversion of malic acid to lactic acid in wines. The advantages of MLF include the deacidification of wine and increased microbiological stability. During this period LAB also produce other aroma and flavour compounds that can influence the final quality of the wine.

Cavin *et al*. (1993) was the first to report the ability of wine LAB to metabolise ferulic acid and *ρ*-coumaric acid. Several strains of LAB isolated from wine were evaluated. It was found that ferulic acid and ρ-coumaric acid was decarboxylated by growing cultures of *Lactobacillus brevis*, *Lactobacillus plantarum* and *Pediococcus pentosaceus* in synthetic media. The decarboxylation activity of *ρ*-coumaric acid by resting cells was

only observed if the bacteria were grown in the presence of this phenolic acid, suggesting inducibility of this metabolism. The decarboxylation activity was twice as high in permeabilised cells than for intact cells possibly indicating that the uptake of *ρ-coumaric* acid by intact cells could be a limiting factor. The decarboxylation activity for *O. oeni* was only observed with permeabilised cells grown in the presence of *ρ*-coumaric acid. This could possibly be due to the absence of a transport system in *O. oeni* for *ρ-coumaric* acid into intact cells. It was also noticed that when the decarboxylation of phenolic acids occurred, 4-ethylguaiacol and 4-ethylphenol were also detected. This indicates the possibility of the reduction step, but the order of this reaction was unknown (Cavin *et al*., 1993). Furthermore Chatonnet *et al*. (1995) found that some strains of *L. brevis* and *P. pentosaceus* were capable of decarboxylating *ρ-coumaric* acid to form 4-vinylphenol in synthetic media. Only *L. plantarum* could produce significant amounts of 4-ethylphenol in a culture media. The concentration of 4-ethylphenol produced by *L. plantarum* is very low in comparison with *D. bruxellensis* and it had no negative impact on the aroma of the wine. They concluded that the contribution of LAB on the production to volatile phenols in wine is very low.

L. plantarum displays a substrate-inducible phenolic acid decarboxylase enzyme activity on phenolic acids, encoded by the *pdc* gene. This gene is transcriptionally regulated by *ρ-coumaric* acid, ferulic acid and caffeic acid (Cavin *et al*., 1997). Cavin *et al*. (1997) found that *L. plantarum* produces at least two different phenolic acid decarboxylases and two specific induction systems. They identified that the presence of *ρ*-coumaric acid induced a specific activity on this acid, but did not show any activity on ferulic acid. Samples supplemented with ferulic acid had a specific activity on *ρ*-coumaric acid and ferulic acid. It was not possible to establish if ferulic acid could induce *ρ*-coumaric acid decarboxylase or if decarboxylase induced by ferulic acid could decarboxylate ferulic acid and *ρ*-coumaric acid. They did however show that *ρ*-coumaric acid decarboxylase (*pdc*) was able to metabolise only *ρ*-coumaric acid and caffeic acid (Cavin *et al*., 1997). Furthermore it has been shown that *L. plantarum* has a second acid phenol decarboxylase enzyme (*pdc2*) that is better induced by ferulic acid than with *ρ-coumaric* acid. *L. plantarum* also displays inducible phenol acid reductase activity (PAR) that is mostly active in the presence of glucose (Barthelmebs *et al*., 2000a). *P. pentosaceus* also displays a substrate-inducible phenolic acid decarboxylase activity on *ρ*-coumaric acid, encoded by the *padA* gene. This lactic acid bacterium have an efficient system to detoxify phenolic acids by organising *padA*, to convert phenolic acids, and *padR*, to regulate the PAD activity, into a single transcriptional unit (Barthelmebs *et al*., 2000b).

In a more recent study LAB, associated with wine, was evaluated for their ability to produce volatile phenols in a culture medium (Couto *et al*., 2006). They found that of the 35 strains, only 37% were able to produce volatile phenols (vinylphenols) from ρ-coumaric acid and only 9% produced 4-ethylphenol. The reduction step was evident only in strains belonging to the *Lactobacillus* genus including *L. brevis*, *L. collinoides* and *L. plantarum*. Most of the *Pediococcus* strains could produce 4-vinylphenol, but was

unable to produce 4-ethylphenol. No volatile phenols were produced from ρ-coumaric acid by the two *O. oeni* strains and the *Leuconostoc mesenteroides* strain. It was found that the ability of LAB to produce volatile phenols from ferulic acid is much lower than from ρ-coumaric acid (Couto *et al*., 2006).

2.8 CONCLUSIONS

Currently the consumer and market preferences dictate the measurement of wine quality. It is therefore extremely important to produce wines that can compete on an international level and comply with consumer requirements. It is clear that the presence of very high levels of volatile phenols leading to phenolic off-flavours in wine is considered to be a negative aroma characteristic. Although other wine microbes can produce volatile phenols in wines, high levels of 4-ethylphenol in wines are still associated with *Brettanomyces* yeast contamination. It is therefore important to understand the factors that influence this spoilage yeast so that possible strategies can be implemented to prevent the contamination of wines, control further spoilage or reduce the levels of volatile phenols in wine. Controlling *Brettanomyces* yeast contamination by implementing the prevention strategies mentioned in this review will also control other microbes that can potentially lead to wine spoilage.

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Chapter 3

RESEARCH RESULTS

Investigating the influence of *Saccharomyces cerevisiae* and malolactic fermentation on the production of volatile phenols by *Brettanomyces bruxellensis*

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3. RESEARCH RESULTS

Investigating the influence of *Saccharomyces cerevisiae* and malolactic fermentation on the production of volatile phenols by *Brettanomyces bruxellensis*

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The production of volatile phenols by *Brettanomyces* during the winemaking process can have a great influence on the aroma of the wine. Therefore the formation of 4-ethylphenol, 4-ethylguaiacol, 4-vinylphenol and 4-vinylguaiacol by *Brettanomyces bruxellensis* B16 was studied under winemaking conditions. The interaction between commercial *Saccharomyces cerevisiae* wine yeast strains and *B. bruxellensis* was investigated in Pinotage and Cabernet Sauvignon juice and wine in 2006 and 2007. It was found that the *S. cerevisiae* WE372 fermentations resulted in higher *Brettanomyces* cell numbers, but did not necessarily lead to higher volatile phenol levels in both varietals studied during both seasons. The *B. bruxellensis* inoculated with *S. cerevisiae* alcoholic fermentations initially had a faster fermentation rate indicating the contribution of *B. bruxellensis* in the conversion of glucose and fructose to alcohol. After alcoholic fermentation *B. bruxellensis* inoculated wines showed slightly higher volatile acidity levels than the control wines without *B. bruxellensis* inoculation for both varietals studied. *Brettanomyces* population densities after malolactic fermentation indicated in some cases that better survival was found if inoculation occurred before the onset of alcoholic fermentation. In the Cabernet Sauvignon varietal after malolactic fermentation higher 4-ethylphenol and 4-ethylguaiacol was found in wines with *B. bruxellensis* inoculation before alcoholic fermentation. There was a tendency for the wines initially fermented with the *S. cerevisiae* WE14 yeast strain to produce higher levels of volatile phenols after malolactic fermentation. There also seem to be a tendency for the spontaneous malolactic fermentation without yeast lees to occasionally lead to higher levels of volatile phenols. The presence of *B. bruxellensis* can also lead to elevated levels of volatile acidity in finished wines.

Keywords: *Brettanomyces bruxellensis*, volatile phenols, *Saccharomyces cerevisiae*, yeast lees, malolactic fermentation

3.1 INTRODUCTION

Wine is the result of the growth and metabolism of a complex mixture of yeasts and bacteria in grape must (Jolly *et al*., 2006). During the process of alcoholic fermentation the conversion of grape sugars (glucose and fructose) to ethanol and carbon dioxide

occurs with the production of various aroma and flavour compounds. Alcoholic fermentation is usually performed by the inoculation of grape juice with commercial *Saccharomyces cerevisiae* wine yeasts strains (Egli *et al*., 1998; Cocolin *et al*., 2002). After alcoholic fermentation most red wines and some white wines undergo a second fermentation known as malolactic fermentation (MLF) (Millet & Lonvaud-Funel, 2000). Lactic acid bacteria (LAB) are responsible for MLF resulting in the conversion of L-malic acid to L-lactic acid in wines. This results in the deacidification of wine and increased microbiological stability. During malolactic fermentation LAB also produce other aroma and flavour compounds that can influence the final quality of the wine. The LAB genera of *Lactobacillus*, *Pediococcus* and *Oenococcus* are mostly associated with wine production (Wibowo *et al*., 1985). Different practices of MLF include the inoculation with pure cultures of *O. oeni* or spontaneous MLF, which is usually performed by a mixture of species from *O. oeni, Lactobacillus* and *Pediococcus*.

Various non-*Saccharomyces* yeasts can also be associated with wine production including species of the genera *Brettanomyces* and its sexual equivalent *Dekkera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora* and its asexual counterpart *Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* (Pretorius *et al*., 1999). These indigenous yeasts can be present on healthy grapes and be transferred to the grape juice where they can have an influence during fermentation. The metabolic activity of these yeasts could lead to the production of metabolites that can have an impact on wine quality. Although these yeasts can have a positive contribution to wine quality, some of them are regarded as microbiological spoilers of wine, especially *Brettanomyces* yeast (Jolly *et al*., 2006).

The first description of *Brettanomyces* yeasts occurred in 1904 by Claussen who associated the characteristic flavour compounds of English stock beer with the presence of a specific non-*Saccharomyces* yeast strain (Gilliland, 1961). *Brettanomyces* spp. have been associated with various fermented products including wine, cider, beer, kombucha and kefyr (Thurston & Tubb, 1981; Licker *et al*., 1998; Arvik *et al.,* 2002; Morrissey *et al*., 2004; Suárez *et al*., 2007). *Brettanomyces bruxellensis* is the species predominantly associated with wine (Fugelsang & Zoecklein, 2003), although *B. anomalus* has also been connected with wine (Cocolin *et al*., 2004).

Wine spoilage by *Brettanomyces* yeast has been reported as being responsible for vast economical losses in the wine industry. Although some winemakers regard the flavour compounds produced by *Brettanomyces* spp. as spoilage, others suggest that the presence of these compounds may contribute to wine complexity. The most common spoilage compounds in wine produced by *Brettanomyces* spp. include volatile phenols, tetrahydropyridines, iso-valeric acid and acetic acid (Heresztyn *et al*., 1986; Chatonnet *et al*., 1992; Boulton *et al*., 1996; Ciani & Ferraro, 1997). It has been stated that *Brettanomyces* yeast can also produce biogenic amines that can affect the wholesomeness of wine (Caruso *et al*., 2002). The possibility also exists that *Brettanomyces* yeast produces other metabolites that could lead to wine spoilage. The

production of volatile phenols is the most important spoilage compound associated with *Brettanomyces* yeast. The most influential volatile phenols include 4-vinylphenol (4-VP), 4-vinylguaiacol (4-VG), 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) (Chatonnet *et al*., 1992). General aroma descriptions of wines with high levels of volatile phenols include phenolic, medicinal, horse sweat, leather, smoky etc. (Chatonnet *et al*., 1992; Rodrigues *et al*., 2001). Recently 4-ethylcatechol has been identified to also contribute to the *Brettanomyces* character of wines (Curtin *et al*., 2007).

Volatile phenols are formed from free hydroxycinnamic acid precursors naturally present in grapes (Chatonnet *et al*., 1992). Hydroxycinnamic acids are usually present in low concentrations in grapes, normally esterified to tartaric acid known as caftaric, coutaric and fertaric acids (Boulton *et al*., 1996; Lao *et al*., 1997). The free form of hydroxycinnamic acids, including: *ρ*-coumaric, ferulic and caffeic acid can be released by certain cinnamoyl esterase activities during the winemaking process (Dugelay *et al*., 1993; Stead, 1995). The action of hydroxycinnamate decarboxylase on *ρ*-coumaric, ferulic or caffeic acid leads to the production of the vinyl derivatives (4-VP and 4-VG) (Edlin *et al*., 1995). Thereafter, the action of vinylphenol reductase reduces the vinyl derivatives to the ethyl derivatives (4-EP and 4-EG) (Dias *et al*., 2003a). Various yeasts found in wine can produce the enzyme that facilitates the decarboxylation step, but the reduction step is mostly associated with *B. bruxellensis*, *B. anomala*, *Pichia guilliermondii*, *Candida versatilis, C. halophila* and *C. mannitofaciens* (Chatonnet *et al*., 1995, 1997; Edlin *et al*., 1995; Dias *et al*., 2003a, b). *Brettanomyces* yeasts show the most active vinylphenol reductase activity and therefore produce the highest concentrations of 4-EP and 4-EG in wine. It has been shown that *S. cerevisiae* yeast strains can produce significant amounts of 4-vinylphenols, but is to date incapable of producing 4-ethylphenols (Chatonnet *et al*., 1993). Van Wyk & Rogers (2000) also found that *S. cerevisiae* strains differed significantly in their ability to produce 4-vinylguaiacol. The production of vinyl derivatives by commercial wine yeast strains could enhance the conversion to the ethyl derivatives by *Brettanomyces* yeasts.

Cavin *et al*. (1993) provided the first results on the ability of LAB, isolated from wine, to decarboxylate ferulic acid and ρ-coumaric acid. They found the decarboxylation step in growing cultures of *Lactobacillus brevis*, *Lactobacillus plantarum*, and *Pediococcus* in synthetic media. Chatonnet *et al*. (1995) also found some strains of *L. brevis* and *P. pentosaceus* were capable of producing significant amounts of 4-VP in synthetic media. The same authors found that *O. oeni* could produce very low levels of 4-VP. With regards to the ethyl derivatives, *L. plantarum* was the only strain that could produce low amounts of 4-EP in culture media. In a more recent study by Couto *et al*. (2006) it was found that *L. brevis*, *L. collinoides* and *L. plantarum* could perform the reduction step to produce 4-EP under laboratory conditions. This can implicate LAB during spontaneous MLF to contribute to higher volatile phenol concentrations in wine. Considering that the cinnamate decarboxylase and vinylphenol reductase enzymes of *B. bruxellensis* are active under oenological conditions this yeast is recognized as a

spoilage microorganism with the potential to produce undesirable levels of ethylphenols during the winemaking process (Chatonnet *et al*., 1995, 1997).

The overriding goal of this study was to generate a better understanding of the effect of different winemaking practises on the production of volatile phenols by *B. bruxellensis*. The first aim was to evaluate the critical stage of winemaking on volatile phenol production. This was done by introducing *B. bruxellensis* at two different stages: in must and before MLF. The second aim was to assess the impact of two different *S. cerevisiae* commercial wine yeast strains performing the alcoholic fermentation process on the volatile phenol levels produced by *B. bruxellensis*. Thirdly, different MLF scenarios (spontaneous vs. inoculated and MLF with or without the presence of yeast lees) were investigated to determine its impact on volatile phenol production by *B. bruxellensis*.

3.2 MATERIALS AND METHODS

3.2.1 SMALL-SCALE WINEMAKING PROCEDURES

Small-scale winemaking of Pinotage and Cabernet Sauvignon grapes were conducted over two vintages (2006 and 2007) to evaluate the influence of different *S. cerevisiae* wine yeast strains and MLF treatments on the production of volatile phenols by *B. bruxellensis* B16, a South African wine isolate. The different treatments were repeated in duplicate in the 2006 season and in triplicate in 2007.

The grapes used in this experiment were obtained from the Paarl, Stellenbosch and Franschoek wine growing regions of South Africa. After 13 kg of grapes were destemmed and crushed, the grape juice and skins were homogenised and equally allocated for each different treatment. SO_2 was added at a concentration of 20 ppm before the onset of alcoholic fermentation to inhibit the growth of indigenous microflora. In the 2007 harvest season the grape must was supplemented with 10 mg/L *ρ*-coumaric acid (Sigma-Aldrich, St Louis, USA), 1 mg/L ferulic acid (Sigma-Aldrich) and 1 mg/L caffeic acid (Sigma-Aldrich) to ensure the presence of precursors for potential volatile phenol production. Before the onset of alcoholic fermentation representative homogeneous samples of the different grape musts were taken to determine the microbiological status and chemical composition. The strains and commercial starter cultures used in this study are listed in **Table 3.1**.

3.2.1.1 Alcoholic fermentation procedures

The grape must was inoculated with commercial strains of *S. cerevisiae*, namely WE14 and WE372 (active dried yeast), at a concentration of $\pm 10^6$ cells/mL. Rehydration and inoculation of the yeast strains was performed according to the manufacturer's specification. Control treatments underwent fermentation only with the specific commercial yeast strain inoculated into the juice. In the other treatments *B. bruxellensis* strain B16 was inoculated at a concentration of $\pm 10^6$ cells/mL after pre-culturing in YPD

medium, into the grape must at the same time as the particular *S. cerevisiae* yeast strain. **Table 3.2** illustrates the different treatments performed during alcoholic fermentation. The decrease in sugar levels was measured daily by using a hydrometer to monitor the progression of alcoholic fermentation. The fermentation was conducted at 25°C and the skins were mixed with the juice twice daily using a manual punch-down method. Diammonium phosphate was added at a concentration of 0.3 g/L on day three of the fermentation process. This aids as a nitrogen source for yeasts to avoid sluggish alcoholic fermentation. At the end of alcoholic fermentation samples were taken to determine the microbial status, chemical composition and volatile phenol levels of the different treatments.

TABLE 3.1.

Yeasts and MLF bacteria used in this study.

Microorganism	**Company**
B. bruxellensis B16	Institute for Wine Biotechnology, Stellenbosch University
S. cerevisiae WE372	Anchor Biotechnology, South Africa
S. cerevisiae WE14	Anchor Biotechnology, South Africa
O. oeni (Lalvin VP41)	Lallemand, South Africa

TABLE 3.2.

Different treatments conducted during alcoholic fermentation for both Pinotage and Cabernet Sauvignon in the 2006 and 2007 harvest season.

Commercial yeast strains used for alcoholic fermentation	***B. bruxellensis* B16 inoculation**
S. cerevisiae WE372	Control – No *B. bruxellensis* inoculated
S. cerevisiae WE372	*B. bruxellensis* co-inoculated with *S. cerevisiae* before alcoholic fermentation
S. cerevisiae WE14	Control – No *B. bruxellensis* inoculated
S. cerevisiae WE14	*B. bruxellensis* co-inoculated with *S. cerevisiae* before alcoholic fermentation

3.2.1.2 Malolactic fermentation procedures

After alcoholic fermentation the wines were pressed with a hydrolic basket press and kept overnight to settle. The wines were then racked from the yeast lees and each of the treatments was divided into four different MLF treatments. The wines were either inoculated with a commercial starter culture (Lalvin VP41) according to the manufacturer's specifications or left to undergo MLF spontaneously. MLF was conducted with or without the presence of yeast lees. The progression of MLF was monitored by the decrease in malic acid measured with FT-MIR spectroscopy (WineScan FT120, FOSS Analytical, Denmark). The control wine from alcoholic

fermentation was divided into two treatments, namely a control with no *B. bruxellensis* addition and in the other treatment *B. bruxellensis* was inoculated at a concentration of $\pm 10^6$ cells/mL, before the onset of MLF. Following MLF completion (malic acid concentration <0.3 g/L) the different treatments were analysed for the microbial status, chemical composition and volatile phenol concentration. **Table 3.3** illustrates the different treatments performed during MLF.

TABLE 3.3.
MLF treatments conducted for Pinotage and Cabernet Sauvignon wines during the 2006 and 2007 vintages (performed in wines initially fermented with either the *S. cerevisiae* WE372 or WE14 strains).

MLF treatment	***B. bruxellensis* B16 inoculation**
MLF spontaneous with yeast lees	Control – No *B. bruxellensis* inoculated
MLF spontaneous without yeast lees	Control – No *B. bruxellensis* inoculated
MLF inoculated with yeast lees	Control – No *B. bruxellensis* inoculated
MLF inoculated without yeast lees	Control – No *B. bruxellensis* inoculated
MLF spontaneous with yeast lees	*B. bruxellensis* inoculation before alcoholic fermentation
MLF spontaneous without yeast lees	*B. bruxellensis* inoculation before alcoholic fermentation
MLF inoculated with yeast lees	*B. bruxellensis* inoculation before alcoholic fermentation
MLF inoculated without yeast lees	*B. bruxellensis* inoculation before alcoholic fermentation
MLF spontaneous with yeast lees	*B. bruxellensis* inoculation after alcoholic fermentation
MLF spontaneous without yeast lees	*B. bruxellensis* inoculation after alcoholic fermentation
MLF inoculated with yeast lees	*B. bruxellensis* inoculation after alcoholic fermentation
MLF inoculated without yeast lees	*B. bruxellensis* inoculation after alcoholic fermentation

3.2.2 MICROBIOLOGICAL ANALYSIS

Microbiological analysis was performed after the following stages: in the juice, after alcoholic fermentation and after MLF. In the case where grape juice was analysed for cell counts, 0.3 g/L biphenyl (Fluka, Steinheim Switzerland) was added to the agar media to prevent mould development. A volume of 100 µL of grape juice or wine (diluted in sterile, de-ionised water in a ten-fold dilution series) was plated on the various selective agar media. The plates were incubated at 30°C between 5 to 14 days depending on the growth of the various microorganisms. The colonies were counted and the cfu/mL was determined. Colonies were inspected by light microscopy to look at the cell morphology.

3.2.2.1 Bacterial species

For the enumeration of *Lactobacillus* and *Pediococcus* bacteria the juice and wine was plated on MRS (Biolab, Merck, South Africa) (De Man Rogosa and Sharp) agar, containing 25 mg/L kanamycin sulphate (Roche, Germany) and 50 mg/L Actistab

(Actistab, Gist-Brocades) (dissolved in 1 mL of sterile dH_2O), to eliminate acetic acid bacteria and yeast growth, respectively. For the specific isolation of *O. oeni* the MRS agar was supplemented with 20 % pure apple juice (preservative free) and the pH adjusted to 5.2 with concentrated HCl. The plates were incubated anaerobically (Oxoid, anaerobic system BR 038B) for 7 days at 30°C before colony counts were performed.

Acetic acid bacteria were enumerated by plating the juice on GYC agar (5% w/v glucose, 1% w/v yeast extract, 3% v/v $CaCO_3$, 2% w/v agar) and the wine on MRS agar (containing 2 % v/v ethanol) and the pH adjusted to 5.0, for both, using concentrated HCl. The media was supplemented with 50 mg/L Actistab and 50 mg/L nisin (dissolved in methanol) to eliminate yeast and lactic acid bacteria, respectively.

3.2.2.2 *Brettanomyces* yeast

Brettanomyces yeast was isolated by plating juice and wine onto WL nutrient agar (Merck, Dramstadt, Germany). The pH of the medium was adjusted to 5.0 and 0.25 g/L sorbic acid (Saarchem-holpro analytic, South Africa), 5 g/L D(+)-trehalose dihydrate (Sigma Aldrich), and 100 mg/L ρ-coumaric acid (Sigma Aldrich) was added to increase the selectivity of this medium. The addition of 50 mg/L cycloheximide (Sigma Aldrich) (dissolved in 1 mL 50% ethanol) eliminated the growth of other yeast species. Lactic acid bacteria and acetic acid bacteria growth on plates was inhibited by 30 mg/L chloramphenicol (Roche, Germany) (dissolved in 1 mL 100 % ethanol) and 25 mg/L kanamycin sulphate (Roche, Germany) (dissolved in 1 mL sterile dH_2O), respectively.

3.2.2.3 *Saccharomyces* and other non-*Saccharomyces* yeast species

For the enumeration of the total yeast population, the juice and wine was plated onto YPD agar (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) (Biolab, Merck, South Africa) supplemented with 25 mg/L kanamycin sulphate and 30 mg/L chloramphenicol to eliminate acetic acid bacteria and lactic acid bacteria, respectively. Non-*Saccharomyces* yeasts were isolated on Lysine agar (lysine medium with 0.01% v/v potassium lactate) (Biolab, Merck, South Africa) supplemented with 25 mg/L kanamycin and 30 mg/L chloramphenicol to prevent acetic acid bacteria and lactic acid bacteria growth, respectively.

3.2.3 STANDARD WINE ANALYSIS

Standard wine analysis of the juice and wine were performed with a WineScan FT 120 spectrophotometer (Foss Analytical, Denmark) (Nieuwoudt *et al*., 2004). The instrument utilises Fourier-transform infrared spectroscopy (FT-MIR). All samples were degassed by successive filtrations using the Filtration Unit (type 70500, Foss Electric, Denmark) with 185 mm diameter filter paper sheets graded at 20-25 µm (Scheicher & Schuell, reference number 10312714) connected to a vacuum pump. This effectively reduced the CO_2 levels to below 300 mg/L, as indicated by the FT-MIR. The standard analysis of the juice included: glucose and fructose concentration, density, total acidity, pH, tartaric acid and malic acid. The standard analysis of the wine included: pH, total acidity, malic

acid, lactic acid, ethanol, residual sugar concentration and volatile acidity. Sulphur dioxide (total and free SO_2 levels) analysis was carried out using the Metrohm titration unit (Metrohm Ltd., Switzerland).

3.2.4 VOLATILE PHENOL ANALYSIS

The analysis included the determination of 4-VP, 4-VG, 4-EP and 4-EG concentrations. During the 2006 harvest season all volatile phenol analysis were conducted by Distell Ltd. The method used for volatile phenol analysis entailed sample preparation by means of ether extraction followed by Gas Chromatography – Flame Ionization Detection (GC-FID) analysis. A volume of 50 µL of the internal standard (200 mg/L 3, 4-dimethylphenol in absolute ethanol) was added to 15 mL of the sample and extracted with 2 mL of diethyl ether (BDH, Lancashire, UK) for 30 minutes on a rotary mixer. A volume of 2 µL of the organic phase was injected into a Hewlett Packard HP 5890 Series II Gas Chromatograph (Avondale, PA, USA) fitted with a flame ionization detector (FID). The column employed was a HP-INNO Wax (60 m x 0.25 mm x 0.5 µm). The carrier gas was hydrogen (pressure: 115 kPa). The injector (split/splitless) was heated to 250°C with a split ratio of 1:20. The oven temperature was held at 50°C for 2 minutes, increased to 150°C at a rate of 10°C/min, where after the temperature was increased to 160°C at a rate of 5°C/min. A final ramp up to 220°C was performed at which the temperature was held for 10 minutes. Volatile phenols were determined quantitatively after a calibration had been set up using standard compounds and the Chemstation chromatography software package (Agilent). Standard compounds for 4-EG, 4-EP and 4-VP were purchased form Sigma-Aldrich (Steinheim, Germany). The 4-VG was purchased from Lancaster Synthesis (Lancaster, UK).

During the 2007 harvest season volatile phenol analyses was conducted by Nanosep Laboratories. Gas Chromatography Mass Spectroscopy (GC-MS) was used to determine the volatile phenol concentration of wines. A liquid-liquid extraction procedure was used by spiking 50 ml of wine with an appropriate internal standard and extraction was done with diethyl ether/hexane. The organic phase was retained and dried over sodium sulphate and then gently concentrated by a low nitrogen flow. Chromatographic analysis were made on a gas chromatograph 3900 equipped with a Saturn 2100T (VARIAN) mass selective detector in a SCAN mode, on a CP WAX 52CB column (30 m x 0.25 mm ID x 0.25 µm film thickness).

3.2.5 STATISTICAL ANALYSIS

Statistical analysis of volatile phenol levels was performed using Statistica version 8 (StatSoft, Inc. 2008, data analysis software system). The statistical analysis of volatile phenol levels after alcoholic fermentation was performed by a two-way analysis of variance and after MLF by repeated measures of ANOVA using a mixed model approach.

3.3 RESULTS

3.3.1 SMALL-SCALE WINEMAKING PROCEDURES BEFORE AND DURING ALCHOLIC FERMENTATION

The chemical composition of the Cabernet Sauvignon and Pinotage grapes used in both 2006 and 2007 are listed in **Table 3.4**. No supplementation of the hydroxycinnamic acids were done in the 2006 vintage, whereas in the 2007 vintage juice was supplemented with additional hydroxycinnamic acids to ensure the presence of precursors for volatile phenol production. **Table 3.5** shows the standard analysis of pH and ethanol after alcoholic fermentation for both varietals, vintages and all the treatments performed. The minimum detection level of the method used for volatile phenol analysis in 2006 was 100 µg/L. Therefore cases graphs that indicate levels below 100 µg/L refer to undetectable levels unless otherwise specified.

TABLE 3.4

Chemical composition of Pinotage and Cabernet Sauvignon musts used for the vinification procedure during 2006 and 2007.

Grape Variety	Growing area	Harvest season	Sugar conc. (g/L)	Total acidity (g/L)	Tartaric acid (g/L)	pH	Malic acid (g/L)
Pinotage	Paarl	2006	245	5.63	4.23	3.53	1.4
Pinotage	Paarl	2007	236	6.11	4.61	3.65	1.5
Cabernet Sauvignon	Stellenbosch	2006	232	6.3	4.8	3.57	1.9
Cabernet Sauvignon	Franschhoek	2007	231	6	4	3.45	2

3.3.1.1 Cabernet Sauvignon 2006

The fermentation performance for the different treatments was measured by the decrease in the sugar concentration (**Figure 3.1**). This was done to determine the influence of *B. bruxellensis* B16 on the alcoholic fermentation rate. In the control treatments the *S. cerevisiae* WE14 strain initially had a faster fermentation rate compared to the *S. cerevisiae* WE372 strain. It is also clear from the fermentation graphs that the treatments inoculated with *B. bruxellensis* B16 initially fermented faster than the control treatments. Although the initial fermentation rates were influenced by the different treatments, all the fermentations finished (below 3 g/L residual sugar) within the same time period.

TABLE 3.5
Ethanol concentrations and pH values of different wines after alcoholic fermentation inoculated with different commercial yeast strains together with or without *B. bruxellensis* contamination.

Grape Variety	Vintage	Commercial yeast strain	Alcoholic fermentation treatment	Ethanol %(v/v)	pH
Cabernet Sauvignon	2006	*S. cerevisiae* WE372	Control	13.20	3.78
		S. cerevisiae WE372	*B. bruxellensis* inoculation before AF	13.19	3.87
		S. cerevisiae WE14	Control	13.16	3.94
		S. cerevisiae WE14	*B. bruxellensis* inoculation before AF	13.19	3.97
Cabernet Sauvignon	2007	*S. cerevisiae* WE372	Control	12.90	3.70
		S. cerevisiae WE372	*B. bruxellensis* inoculation before AF	12.66	3.74
		S. cerevisiae WE14	Control	12.92	3.92
		S. cerevisiae WE14	*B. bruxellensis* inoculation before AF	13.18	3.95
Pinotage	2006	*S. cerevisiae* WE372	Control	16.00	3.85
		S. cerevisiae WE372	*B. bruxellensis* inoculation before AF	16.04	3.91
		S. cerevisiae WE14	Control	16.25	3.93
		S. cerevisiae WE14	*B. bruxellensis* inoculation before AF	16.35	3.95
Pinotage	2007	*S. cerevisiae* WE372	Control	16.40	3.93
		S. cerevisiae WE372	*B. bruxellensis* inoculation before AF	16.38	3.93
		S. cerevisiae WE14	Control	16.57	4.00
		S. cerevisiae WE14	*B. bruxellensis* inoculation before AF	16.21	4.00

Microbiological analysis showed that *B. bruxellensis* B16 could survive during alcoholic fermentation conducted by both the *S. cerevisiae* strains used during this study (**Figure 3.2**). For the Cabernet Sauvignon 2006 there was a significant reduction in the *B. bruxellensis* B16 population from the inoculation level of 10^6 cells/mL to approximately 10^2 cells/mL after alcoholic fermentation, but it is evident that these yeasts could survive during this process. It is also clear from **Figure 3.2** that the total culturable *Brettanomyces* spp. population was higher with the alcoholic fermentation performed by *S. cerevisiae* WE372 yeast strain.

The volatile acidity of the wines after alcoholic fermentation was measured by the WineScan FT 120 spectrometer (Foss Analytical, Denmark). **Figure 3.3** clearly shows the influence of the commercial *S. cerevisiae* yeast strains and *B. bruxellensis* B16 inoculation before alcoholic fermentation on the volatile acidity. Higher concentrations were obtained in wines inoculated with *B. bruxellensis* B16 before alcoholic fermentation. There is a tendency for the *S. cerevisiae* WE372 performed alcoholic fermentations to contain slightly higher levels of volatile acidity.

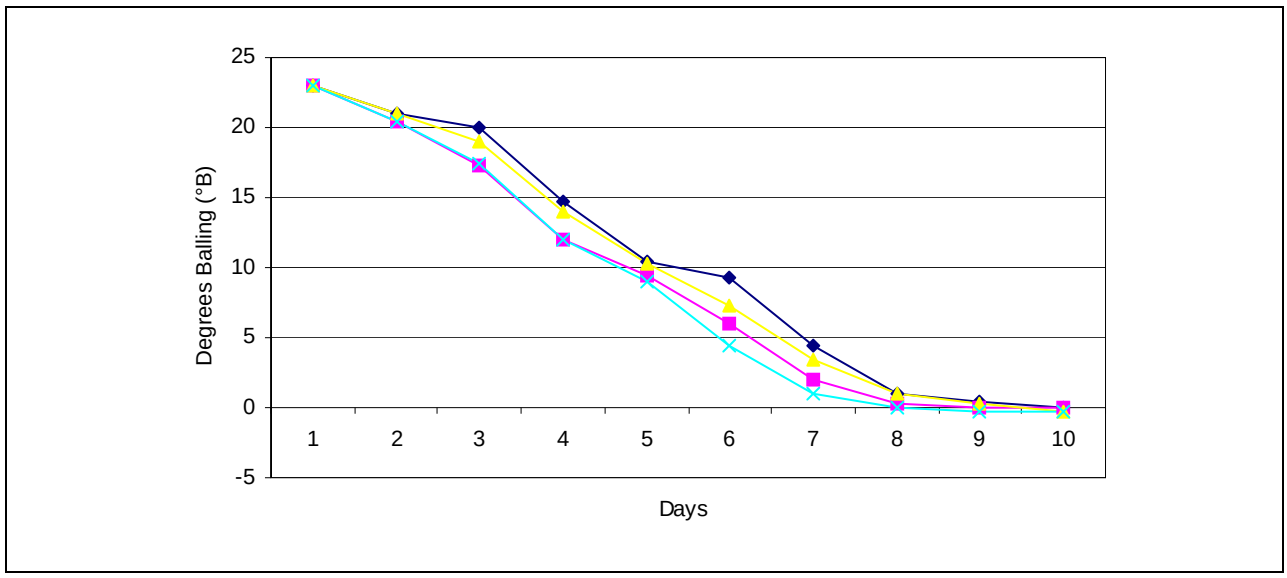

FIGURE 3.1
Fermentation graphs of the *S. cerevisiae* WE372 and WE14 strains performed during the 2006 vintage in Cabernet Sauvignon juice for control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation (*S. cerevisiae* WE372 ♦, *S. cerevisiae* WE14 ■, co-inoculation *S. cerevisiae* WE372 and *B. bruxellensis* B16 ▲, co-inoculation *S. cerevisiae* WE372 and *B. bruxellensis* B16 X). Data shown indicates the average fermentation rates of each treatment repeated in duplicate.

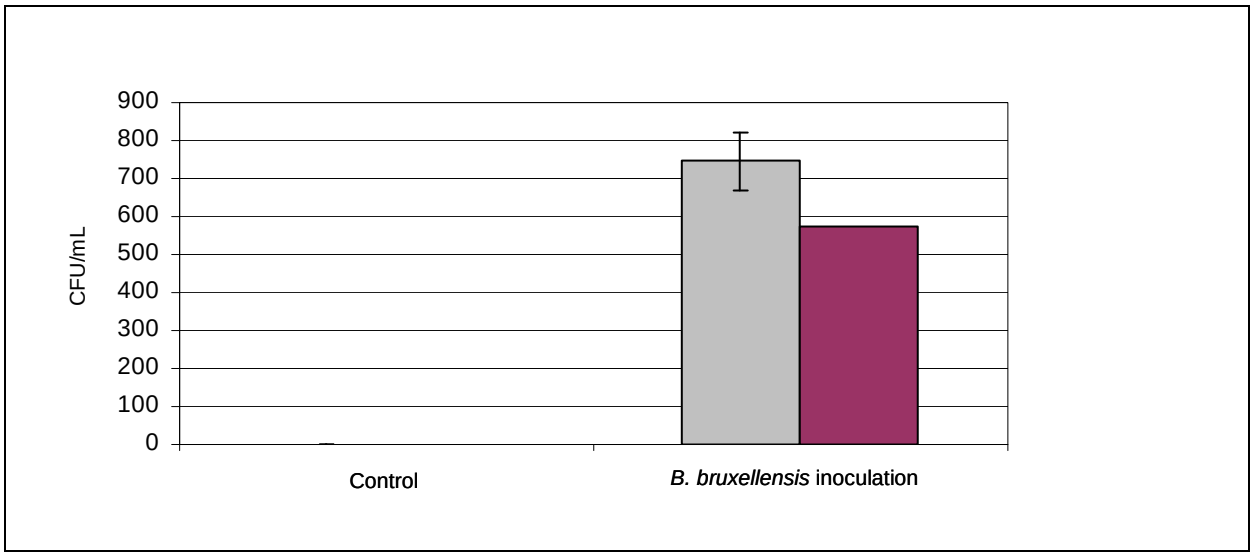

FIGURE 3.2
Brettanomyces spp. cell counts after alcoholic fermentation in the 2006 Cabernet Sauvignon wines fermented with *S. cerevisiae* WE372 (■) and *S. cerevisiae* WE14 (■) strains for the control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. The colony forming units represent the average of duplicates for each treatment.

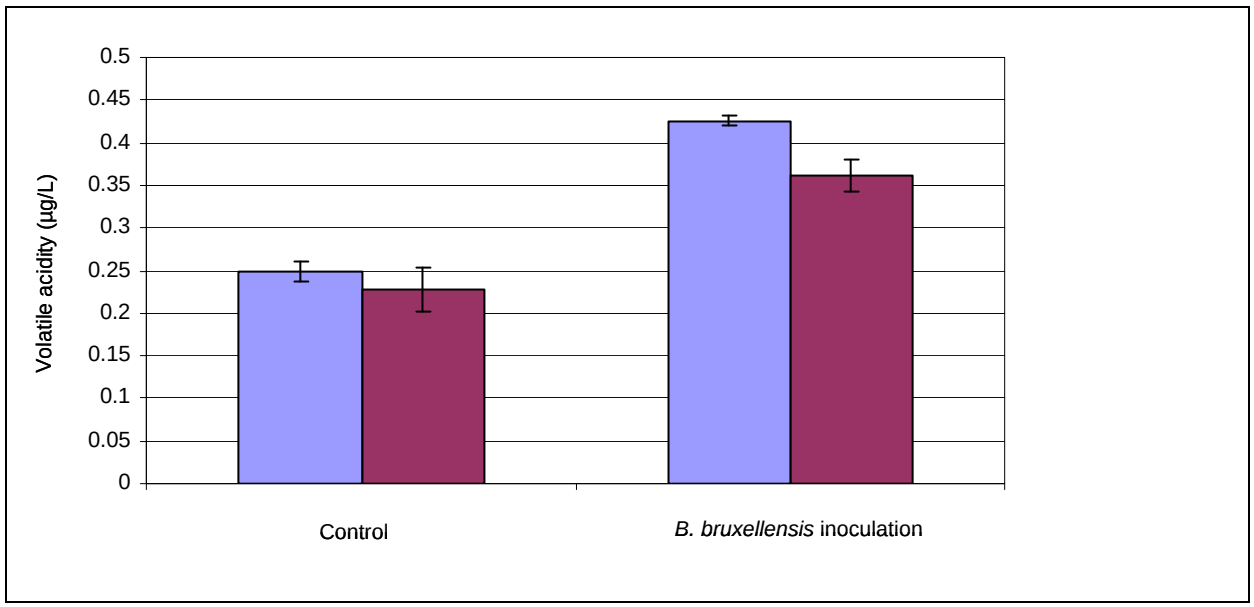

FIGURE 3.3
Volatile acidity concentrations after alcoholic fermentation in Cabernet Sauvignon 2006 wines fermented with *S. cerevisiae* WE372 (■) and *S. cerevisiae* WE14 (■) strains for the control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. Error bars indicate the standard deviation for experiments done in duplicate.

The production of the different volatile phenols (4-EP, 4-EG, 4-VP and 4-VG) was measured after alcoholic fermentation in wines fermented with *S. cerevisiae* WE372 and *S. cerevisiae* WE14 strains, for the control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. **Figure 3.4** illustrates the 4-VP concentrations produced during alcoholic fermentation of all the treatments and significantly higher levels was measured for the *S. cerevisiae* WE14 ($p<0.01$) compared to the *S. cerevisiae* WE372 control fermentations. *B. bruxellensis* B16 inoculation before alcoholic fermentation yielded lower levels of 4-VP after alcoholic fermentation conducted by the *S. cerevisiae* WE14 yeast strain. Very low levels of 4-VG was produced during the *S. cerevisiae* WE14 control fermentations and no detectable levels were obtained in the other treatments (data not shown).

Significantly higher concentrations of 4-EP was produced during both the *S. cerevisiae* WE372 and *S. cerevisiae* WE14 fermentations in treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation (**Figure 3.5**) ($p<0.01$). The concentration of 4-EP produced by *B. bruxellensis* B16 was much higher than the aroma threshold of 440-600 µg/L (Chatonnet *et al*., 1995). No significant differences were found in the levels of 4-EP produced during the alcoholic fermentation treatments with the different commercial *S. cerevisiae* yeast strains co-inoculated with *B. bruxellensis* ($p=0.4$).

The concentration of 4-EG (data not shown) was also significantly higher in the treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation ($p<0.01$). The *B. bruxellensis* B16 inoculated treatments also contained levels of 4-EG (approximately 300-350 µg/L) above the sensory threshold of 110 µg/L for red wines

(Chatonnet *et al*., 1995). No significant differences in the levels of 4-EG were obtained during the fermentation by the different commercial *S. cerevisiae* yeast strains (p=0.2).

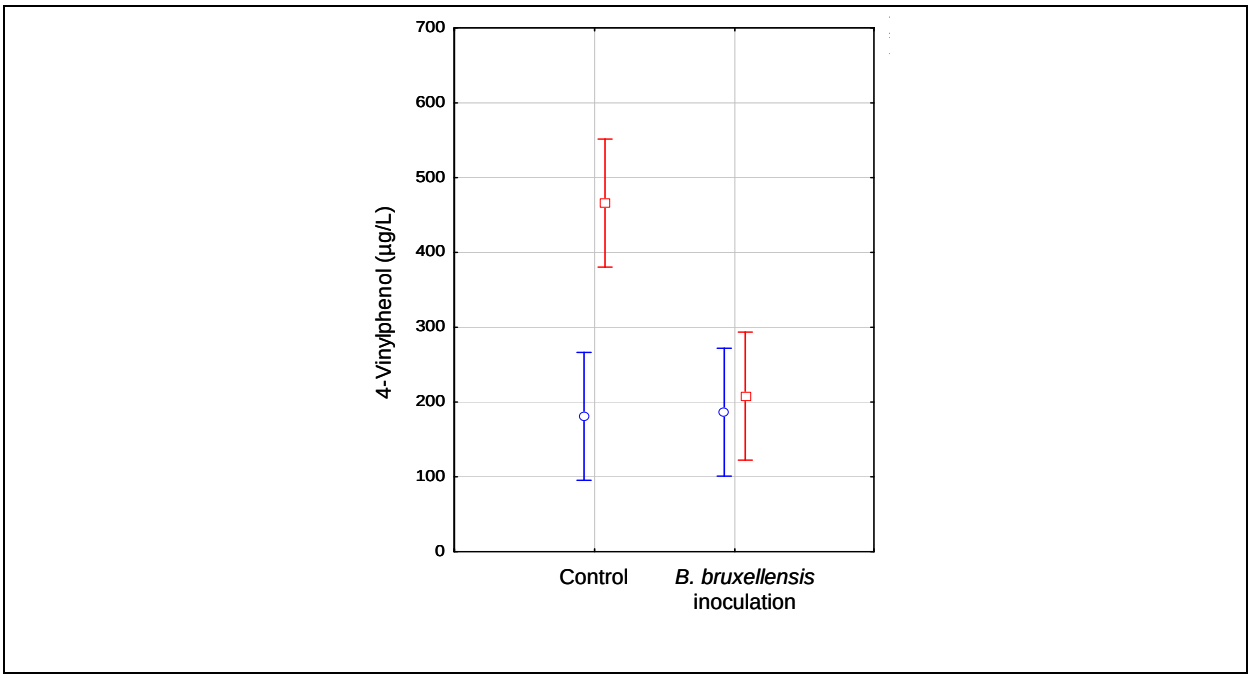

FIGURE 3.4
4-Vinylphenol production during alcoholic fermentation treatments in the Cabernet Sauvignon 2006 fermented with *S. cerevisiae* WE372 (—) and *S. cerevisiae* WE14 (—) strains in the control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. Vertical bars denote a 0.95 confidence interval obtained for 4-VP of duplicate treatments.

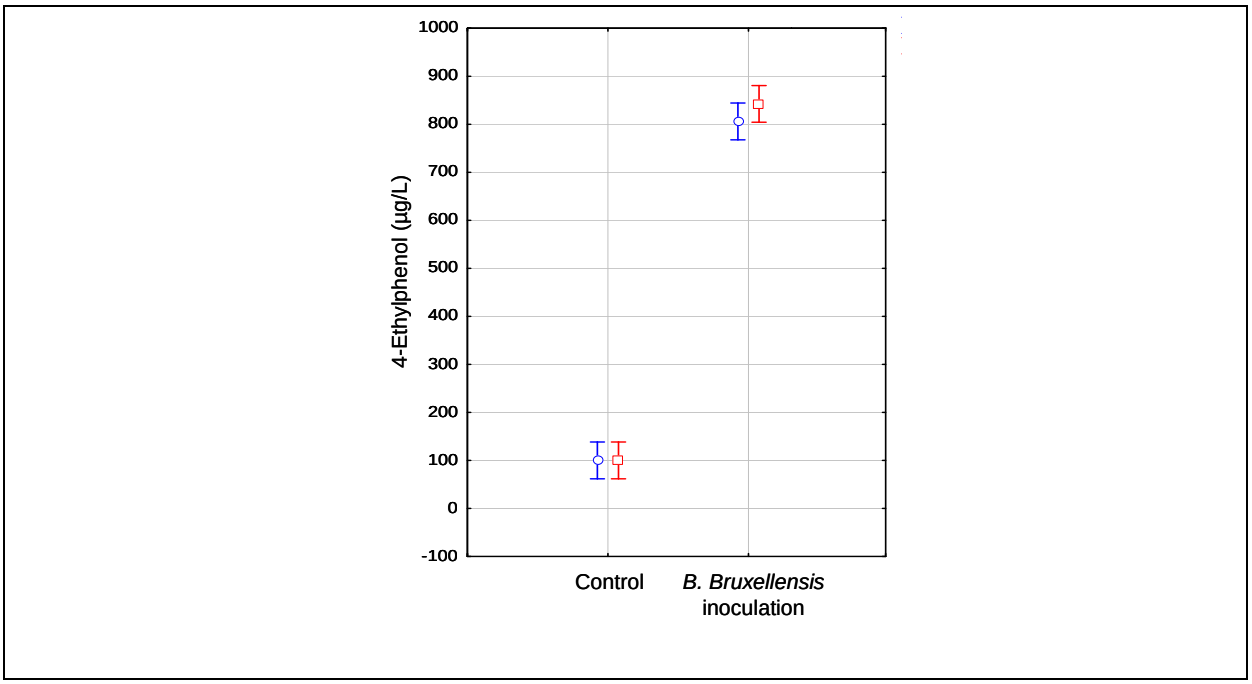

FIGURE 3.5
4-Ethylphenol production during alcoholic fermentation treatments in the Cabernet Sauvignon 2006 fermented with *S. cerevisiae* WE372 (—) and *S. cerevisiae* WE14 (—) strains in the control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. Vertical bars denote a 0.95 confidence interval obtained for 4-EP of duplicate treatments.

3.3.1.2 Pinotage 2006

During the alcoholic fermentation the Pinotage 2006 showed the same tendency than the Cabernet Sauvignon 2006 with regard to the fermentation rate, *Brettanomyces* colony forming units and volatile acidity produced in the wine. The initial fermentation rate was also faster in the treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation (data not shown). In this wine it is also evident that *B. bruxellensis* yeast could survive during the alcoholic fermentation and the reduction in the total *Brettanomyces* spp. population was lower than the Cabernet Sauvignon 2006. In this case the *S. cerevisiae* WE372 fermentations also allowed better culturability of *Brettanomyces* spp. There was no difference in the initial fermentation rate between the commercial *S. cerevisiae* yeast strains (data not shown). The volatile acidity of the *B. bruxellensis* B16 inoculated wines was slightly higher than the control treatments and there was also a tendency of the *S. cerevisiae* WE372 fermentations to have higher volatile acidity levels (data not shown).

The levels of volatile phenols in the Pinotage 2006 were very low and only 4-VP was present in detectable amounts after alcoholic fermentation with the *S. cerevisiae* WE14 strain (**Figure 3.6**). There also seems to be a tendency for the treatment inoculated with *B. bruxellensis* B16 and fermented with *S. cerevisiae* WE14 to have higher levels of 4-VP.

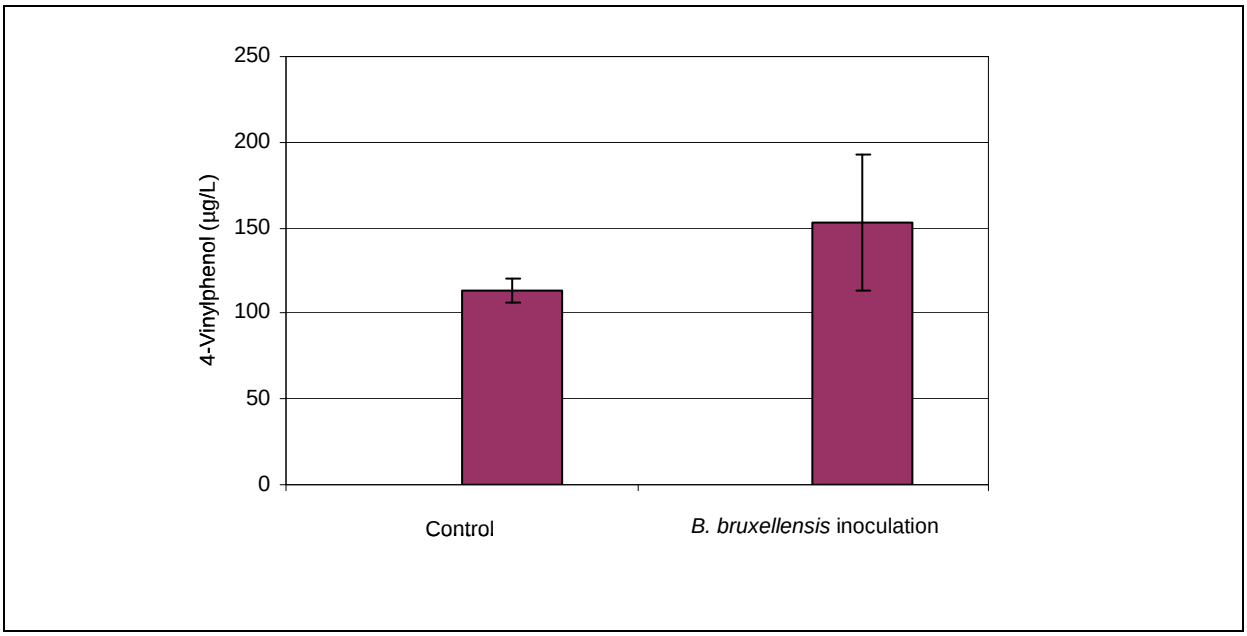

FIGURE 3.6
4-Vinylphenol production during alcoholic fermentation treatments in Pinotage 2006 wines fermented with *S. cerevisiae* WE372 (■) and *S. cerevisiae* WE14 (■) strains in the control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. The concentrations represent the averages of treatments performed in duplicate.

3.3.1.3 Cabernet Sauvignon 2007

During the alcoholic fermentation of the Cabernet Sauvignon 2007 similar tendencies was shown as the Cabernet Sauvignon 2006 with regard to the fermentation rate, *Brettanomyces* spp. colony forming units and volatile acidity produced in the wine. There was a slight difference in the fermentation rate with a noticeable extended lag phase before the onset of fermentation. This did not influence the time it took to finish the fermentation and these wines had lower residual sugar levels after alcoholic fermentation (data not shown). The *Brettanomyces* cell counts were also higher after the *S. cerevisiae* WE372 fermentations for the treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation (**Figure 3.7**). The total reduction in the *Brettanomyces* population was much lower (from approximately 10^6 to 10^4 cells/mL) in comparison to the Cabernet Sauvignon 2006. This indicates better survival of *B. bruxellensis* B16 during the alcoholic fermentation of Cabernet Sauvignon 2007. The Cabernet Sauvignon 2007 treatments inoculated with *B. bruxellensis* B16 had higher levels of volatile acidity compared to the control treatments (similar tendencies than the Cabernet Sauvignon 2006), but no differences were observed in the levels produced during the fermentation with the different commercial *S. cerevisiae* strains (data not shown).

The production of volatile phenols was higher in the Cabernet Sauvignon 2007 compared to the Cabernet Sauvignon 2006 indicating that the supplementation of must with hydroxycinnamic acid precursors yielded higher levels of volatile phenols. No significant differences was observed in the production of 4-VP ($p=0.3$), 4-VG ($p=0.3$), 4-EP ($p=0.8$) and 4-EG ($p=0.03$) between the treatments fermented with either the *S. cerevisiae* WE372 strain or the *S. cerevisiae* WE14 strain. The treatments inoculated with *B. bruxellensis* B16 did not have significantly higher or lower levels of 4-VP ($p=0.1$) and 4-VG ($p=0.02$), although there is a tendency for these treatments to have lower 4-VP and 4-VG levels (data not shown). **Figures 3.8** & **3.9** clearly indicates the significant differences in the levels of 4-EP ($p<0.01$) and 4-EG ($p<0.01$) produced in the control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation, regardless the commercial *S. cerevisiae* yeast strain used to perform the fermentation. The levels of 4-EP and 4-EG produced were much higher than the aroma threshold values in wine.

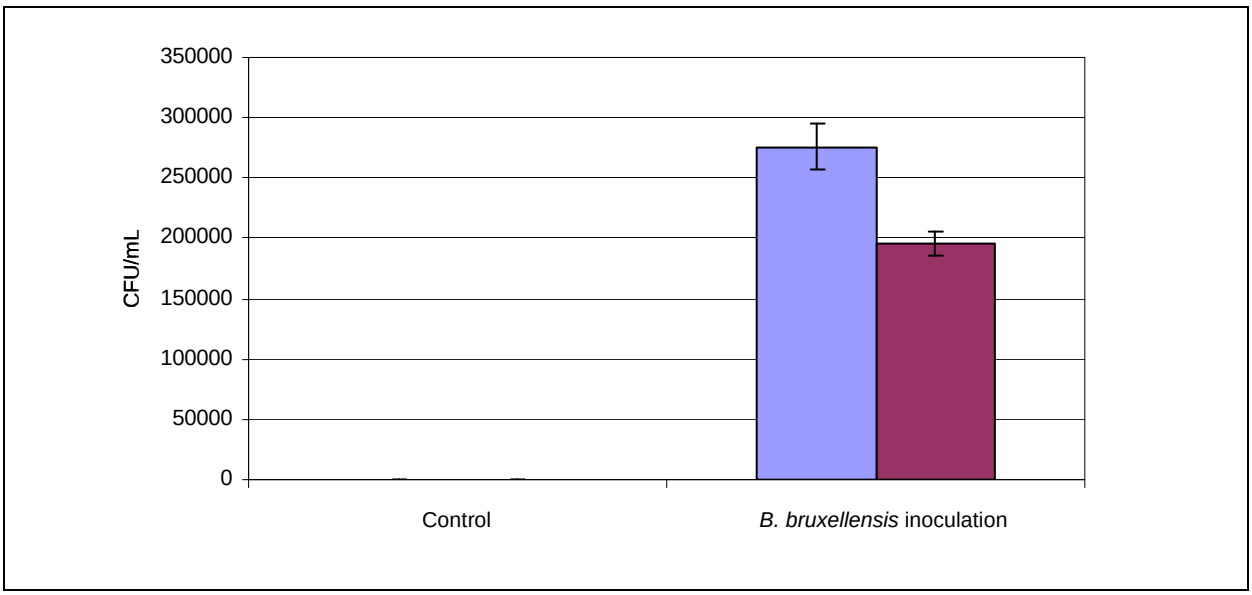

FIGURE 3.7
Brettanomyces spp. colony forming units after alcoholic fermentation in the 2007 Cabernet Sauvignon wines fermented with *S. cerevisiae* WE372 (■) and *S. cerevisiae* WE14 (■) strains for the control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. The colony forming units represent the average of the triplicates for each treatment.

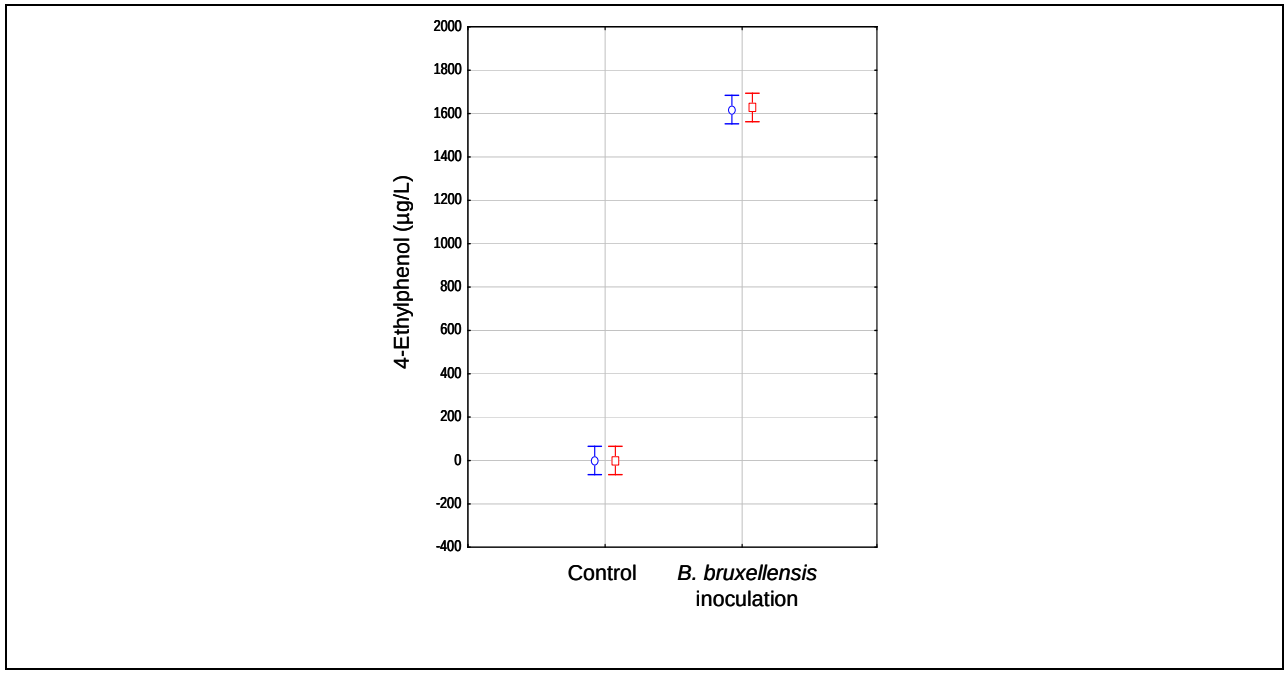

FIGURE 3.8
4-Ethylphenol production during alcoholic fermentation treatments in the Cabernet Sauvignon 2007 fermented with *S. cerevisiae* WE372 (—) and *S. cerevisiae* WE14 (—) strains in the control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. Vertical bars denote a 0.95 confidence interval obtained for 4-EP of triplicate treatments.

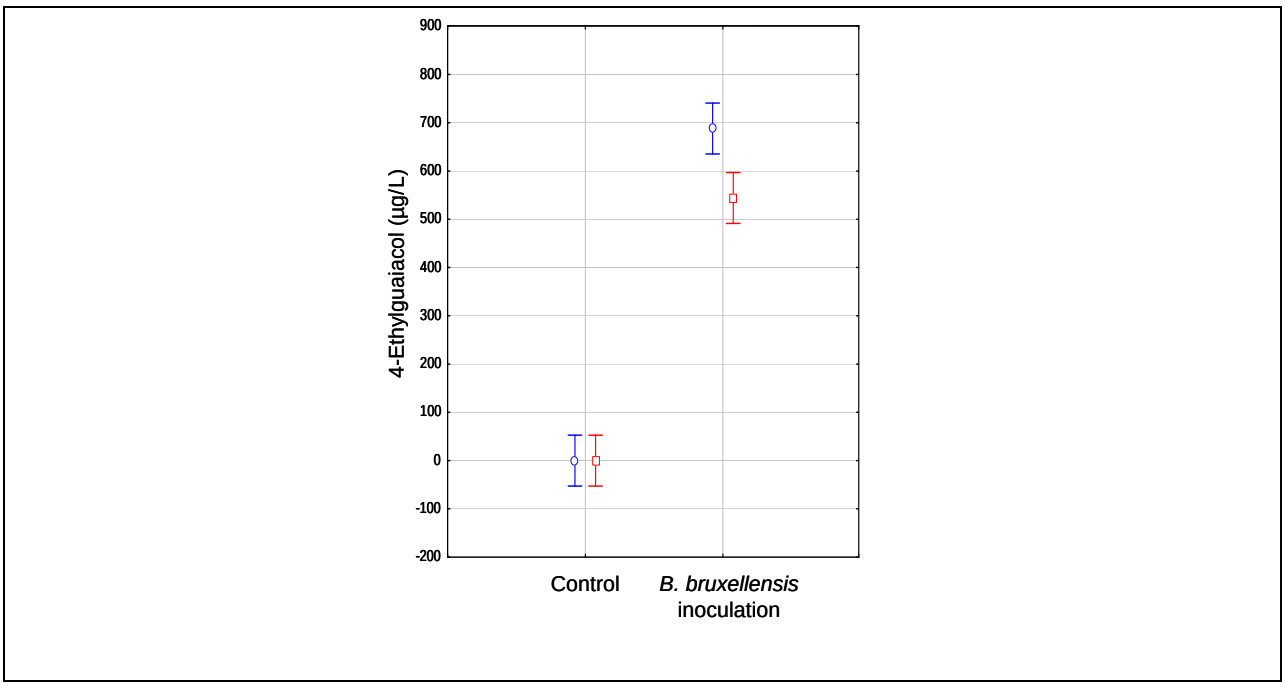

FIGURE 3.9
4-Ethylguaiacol production during alcoholic fermentation treatments in the Cabernet Sauvignon 2007 fermented with *S. cerevisiae* WE372 (—) and *S. cerevisiae* WE14 (—) strains in the control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. Vertical bars denote a 0.95 confidence interval obtained for 4-EG of triplicate treatments.

3.3.1.4 Pinotage 2007

The alcoholic fermentation rate and *Brettanomyces* spp. population showed similar tendencies than the Cabernet Sauvignon 2006 (data not shown). The treatments inoculated with *B. bruxellensis* B16 had higher volatile acidity levels. Results also showed a tendency for the *S. cerevisiae* WE372 fermentations to produce slightly higher volatile acidity levels (data not shown).

There was a tendency for the wines fermented with *S. cerevisiae* WE14 ($p<0.05$), regardless if *B. bruxellensis* B16 inoculation occurred or not to have higher levels of 4-VP (**Figure 3.10**). Significantly higher levels of 4-VG were produced during the *S. cerevisiae* WE14 fermentations ($p<0.01$) (**Figure 3.11**). **Figures 3.12** & **3.13** clearly indicates the significantly higher levels of 4-EP and 4-EG produced during alcoholic fermentation for the treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation ($p<0.01$). Significantly higher levels of 4-EP and 4-EG was also produced in the wines fermented with *S. cerevisiae* WE372 for the treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation ($p<0.01$). No 4-EP and 4-EG was produced in the control wines.

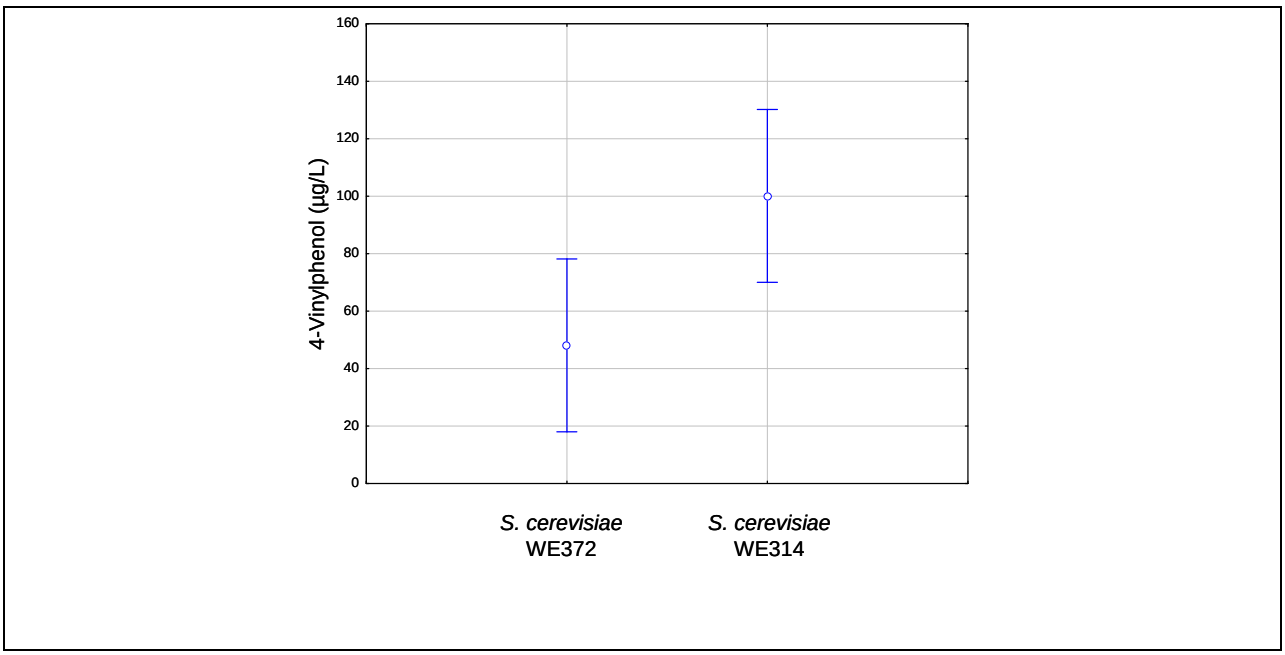

FIGURE 3.10
4-Vinylphenol production in Pinotage 2007 during alcoholic fermentation by the different commercial *S. cerevisiae* yeast strains. Vertical bars denote a 0.95 confidence interval obtained for 4-VP of triplicate treatments.

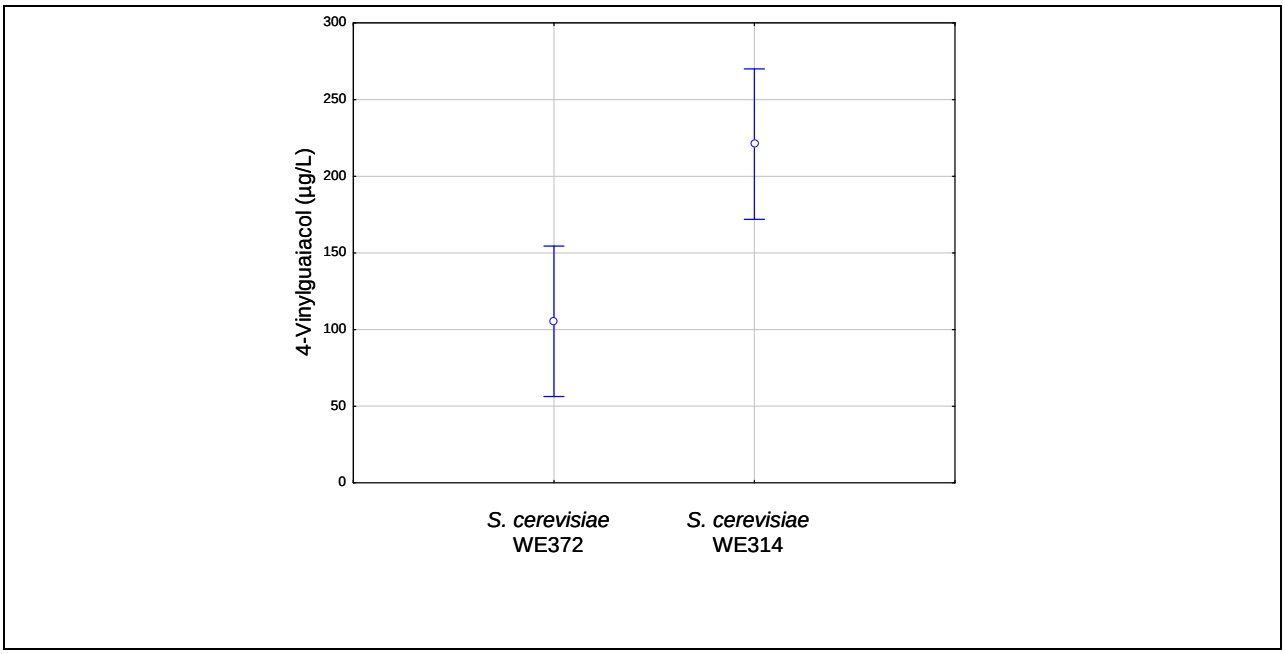

FIGURE 3.11
4-Vinylguaiacol production in Pinotage 2007 during alcoholic fermentation by the different commercial *S. cerevisiae* yeast strains. Vertical bars denote a 0.95 confidence interval obtained for 4-VG of triplicate treatments.

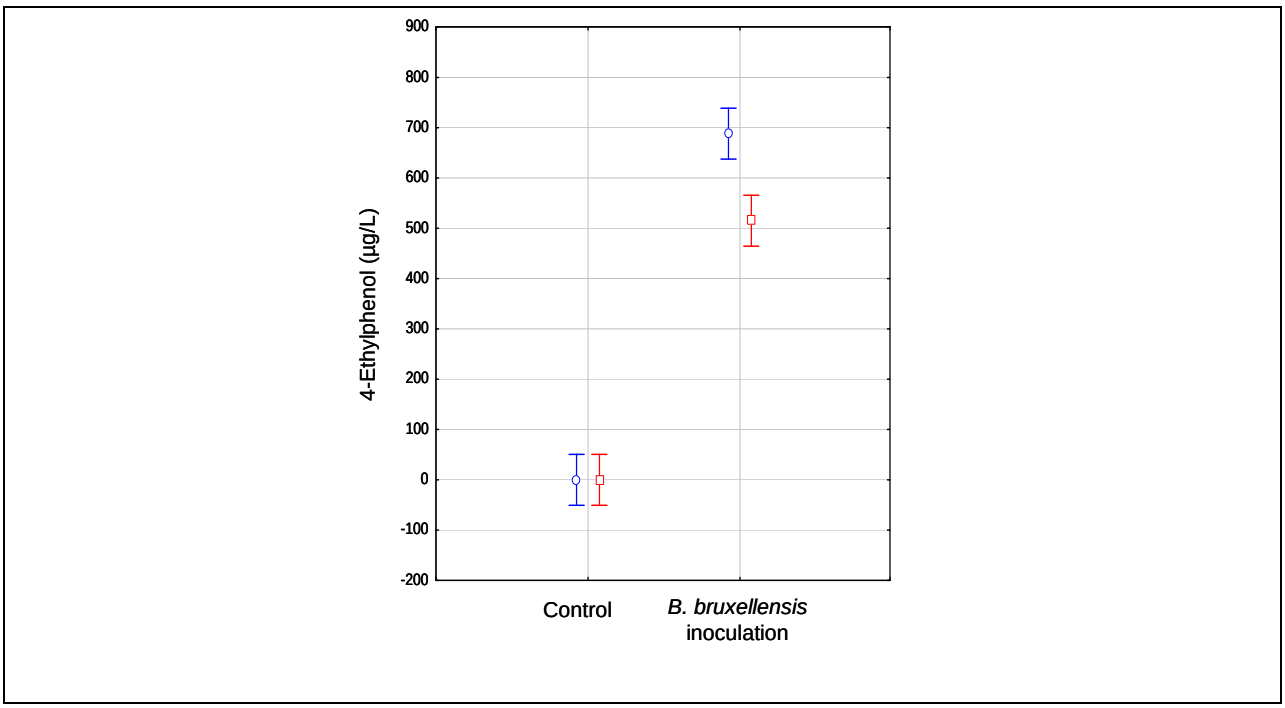

FIGURE 3.12
4-Ethylphenol production during alcoholic fermentation treatments in the Pinotage 2007 fermented with *S. cerevisiae* WE372 (—) and *S. cerevisiae* WE14 (—) strains in the control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. Vertical bars denote a 0.95 confidence interval obtained for 4-EP of triplicate treatments.

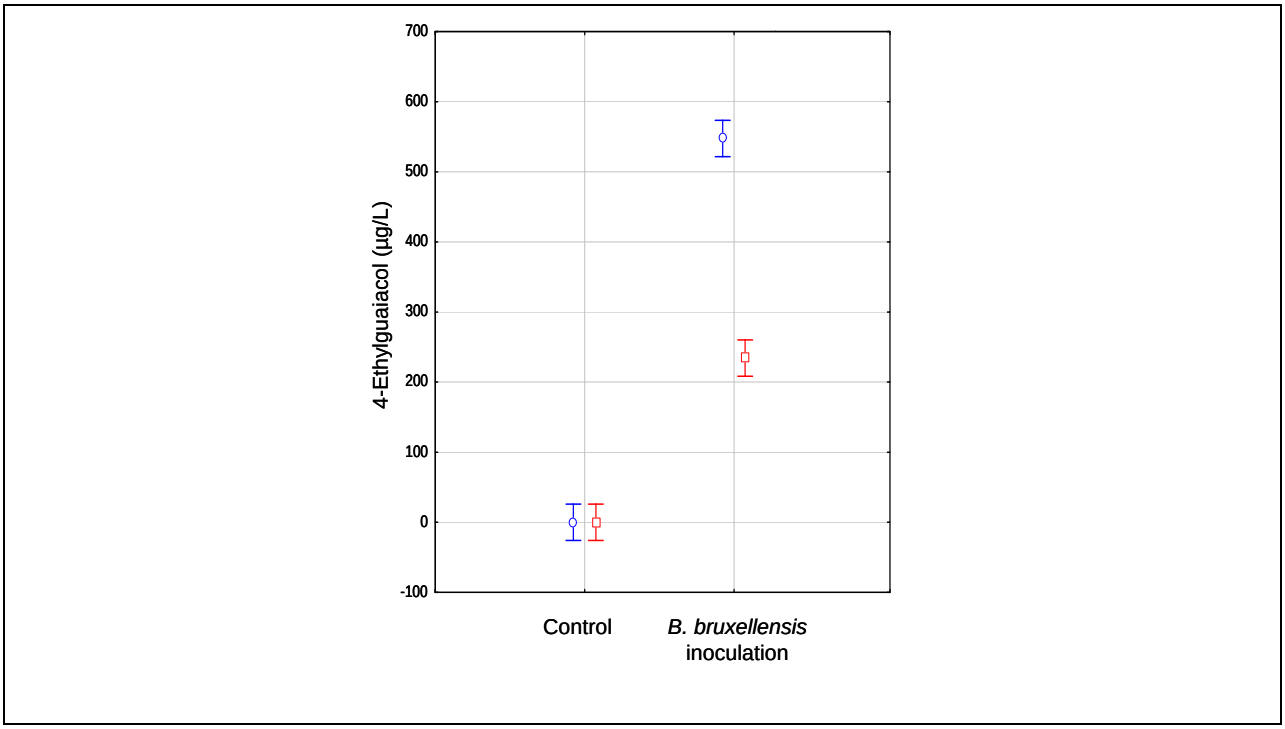

FIGURE 3.13
4-Ethylguaiacol production during alcoholic fermentation treatments in the Pinotage 2007 fermented with *S. cerevisiae* WE372 (—) and *S. cerevisiae* WE14 (—) strains in the control treatments and treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. Vertical bars denote a 0.95 confidence interval obtained for 4-EG of triplicate treatments.

3.3.2 SMALL-SCALE WINEMAKING PROCEDURES AFTER ALCHOLIC FERMENTATION

During the 2006 and 2007 vintages the growth and survival of *B. bruxellensis* B16 were determined after different MLF treatments for the Cabernet Sauvignon and Pinotage varietals. MLF was successfully completed in all the varietals, vintages and treatments performed. The duration of inoculated and spontaneous MLF was similar during both varietals and vintages studied. Standard analysis of the wine (data not shown) was conducted after the completion of MLF. The volatile acidity levels were also measured and it was found that slightly higher levels were found in treatments inoculated with *B. bruxellensis* B16 before or after alcoholic fermentation (data not shown).

3.3.2.1 Cabernet Sauvignon 2006

After the completion of malolactic fermentation the *Brettanomyces* yeast cell numbers were monitored to determine the effect of different MLF practices on *B. bruxellensis* growth and survival. **Figure 3.14** illustrates the *Brettanomyces* cell numbers after MLF in the control treatments and treatments inoculated with *B. bruxellensis* B16 before and after alcoholic fermentation initially fermented with *S. cerevisiae* WE372. The total culturable *Brettanomyces* population was higher in the treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. No *Brettanomyces* populations was detected in the control treatments and treatments inoculated with *B. bruxellensis* B16 after alcoholic fermentation. The different MLF treatments performed also had an effect on the total culturable *Brettanomyces* population with the highest levels found with inoculated MLF without yeast lees followed by low levels with MLF inoculated with yeast lees. No culturable *Brettanomyces* populations were obtained with spontaneous MLF with or without yeast lees. **Figure 3.15** illustrates the *Brettanomyces* cell numbers after MLF in the control treatments and treatments inoculated with *B. bruxellensis* B16 before and after alcoholic fermentation initially fermented with *S. cerevisiae* WE14. The total culturable *Brettanomyces* population was also higher in the treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. Slight contamination did occur in the control treatments and low levels of culturable *Brettanomyces* spp. could be detected in treatments inoculated with *B. bruxellensis* B16 after alcoholic fermentation. MLF inoculated without yeast lees had the highest culturable *Brettanomyces* population followed by MLF spontaneous without yeast lees. There was also a tendency for *S. cerevisiae* WE14 fermentations to contain higher culturable *Brettanomyces* populations compared to the *S. cerevisiae* WE372 fermentations after malolactic fermentation.

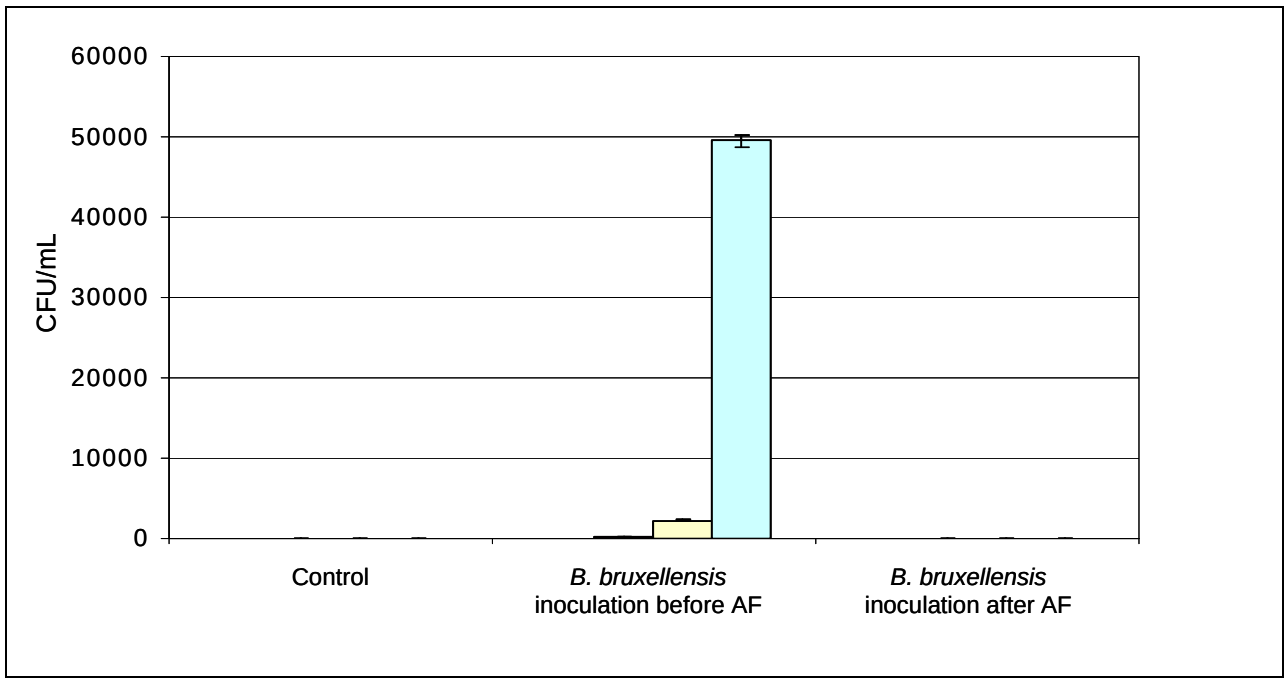

FIGURE 3.14
Brettanomyces spp. colony forming units after malolactic fermentation in the 2006 Cabernet Sauvignon wines fermented with *S. cerevisiae* WE372 strains for the control treatments and treatments inoculated with *B. bruxellensis* B16 before or after alcoholic fermentation (MLF spontaneous with yeast lees ■, MLF spontaneous without yeast lees ■, MLF inoculated with yeast lees ■ and MLF inoculated without yeast lees ■). The colony forming units represent the average of triplicate for each treatment.

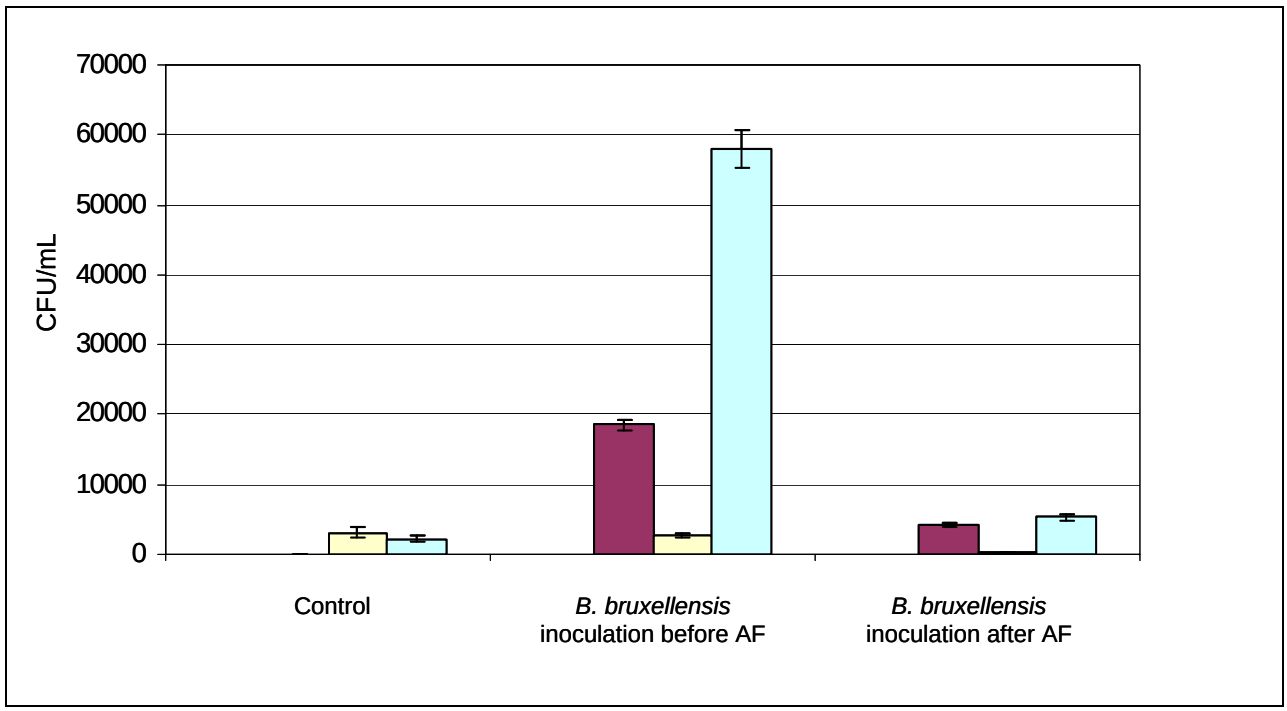

FIGURE 3.15
Brettanomyces spp. colony forming units after malolactic fermentation in the 2006 Cabernet Sauvignon wines fermented with *S. cerevisiae* WE14 strains for the control treatments and treatments inoculated with *B. bruxellensis* B16 before or after alcoholic fermentation (MLF spontaneous with yeast lees ■, MLF spontaneous without yeast lees ■, MLF inoculated with yeast lees ■ and MLF inoculated without yeast lees ■). The colony forming units represent the average of triplicate for each treatment.

The levels of 4-VP in wines after MLF for the different treatments preformed is illustrated in **Figures 3.16** & **3.17**. No significant differences could be obtained in the 4-VP levels in wines after spontaneous or inoculated MLF. There is a tendency for the control treatment to have higher levels of 4-VP compared to the treatment inoculated with *B. bruxellensis* B16 before alcoholic fermentation (p=0.03) (**Figure 3.16**). **Figure 3.17** clearly indicates the differences in the treatments initially fermented with different commercial *S. cerevisiae* yeast strains and MLF conducted with or without yeast lees. The
S. cerevisiae WE14 fermented wine in MLF with yeast lees treatment had higher 4-VP levels. There was also a tendency for the *S. cerevisiae* WE14 fermented wine to have higher
4-VG levels after MLF (p=0.09) (data not shown). **Figures 3.18** & **3.19** illustrates the levels of 4-EP in wines after spontaneous and inoculated MLF treatments performed with or without yeast lees in control treatments or treatments inoculated with *B. bruxellensis* before or after alcoholic fermentation in wines initially fermented with different commercial *S. cerevisiae* yeast strains. Significant differences were shown between the control treatments and treatments inoculated with *B. bruxellensis* B16 before or after alcoholic fermentation ($p<0.01$). Much higher levels of 4-EP was found in wines after MLF for treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. These levels are also higher than the aroma threshold values and would therefore influence the aroma profile of the wines. No detectable levels of 4-EP were found in the control treatments and very low levels (mostly undetected) in the treatments inoculated with *B. bruxellensis* B16 after alcoholic fermentation. There are also clearly significant differences in the treatments initially fermented with different commercial *S. cerevisiae* yeast strains, with higher levels of 4-EP in *S. cerevisiae* WE14 compared to the *S. cerevisiae* WE372 fermentations. There is also a tendency for the treatments with yeast lees to have higher levels of 4-EP ($p<0.05$). **Figure 3.20** indicates that significantly higher levels of 4-EG could be found in wines inoculated with *B. bruxellensis* before alcoholic fermentation ($p<0.01$). The *S. cerevisiae* WE14 also had significantly higher levels of 4-EG compared to the *S. cerevisiae* WE372 fermented wine in the treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation ($p<0.01$). There was also a tendency for higher levels of 4-EG in spontaneous MLF treatments (**Figure 3.21**), but no significant differences were obtained between MLF with or without yeast lees.

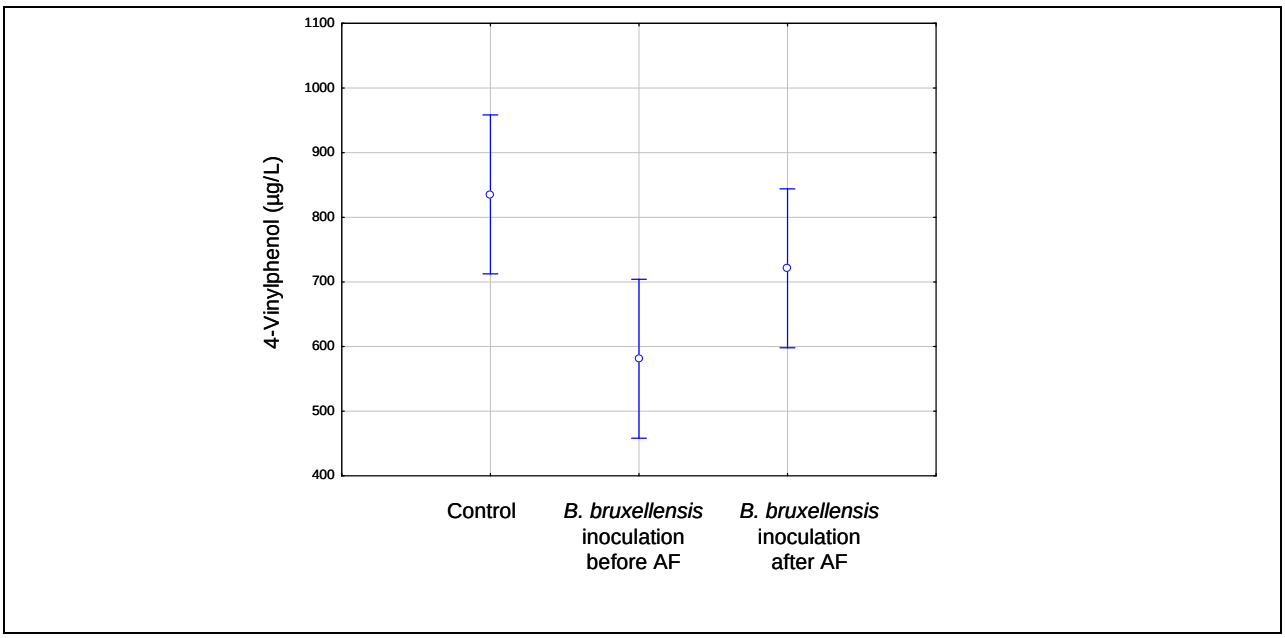

FIGURE 3.16
4-Vinylphenol production in Cabernet Sauvignon 2006 after malolactic fermentation for control treatments and treatments inoculated with *B. bruxellensis* before or after alcoholic fermentation. Vertical bars denote a 0.95 confidence interval obtained for 4-VP of duplicate treatments.

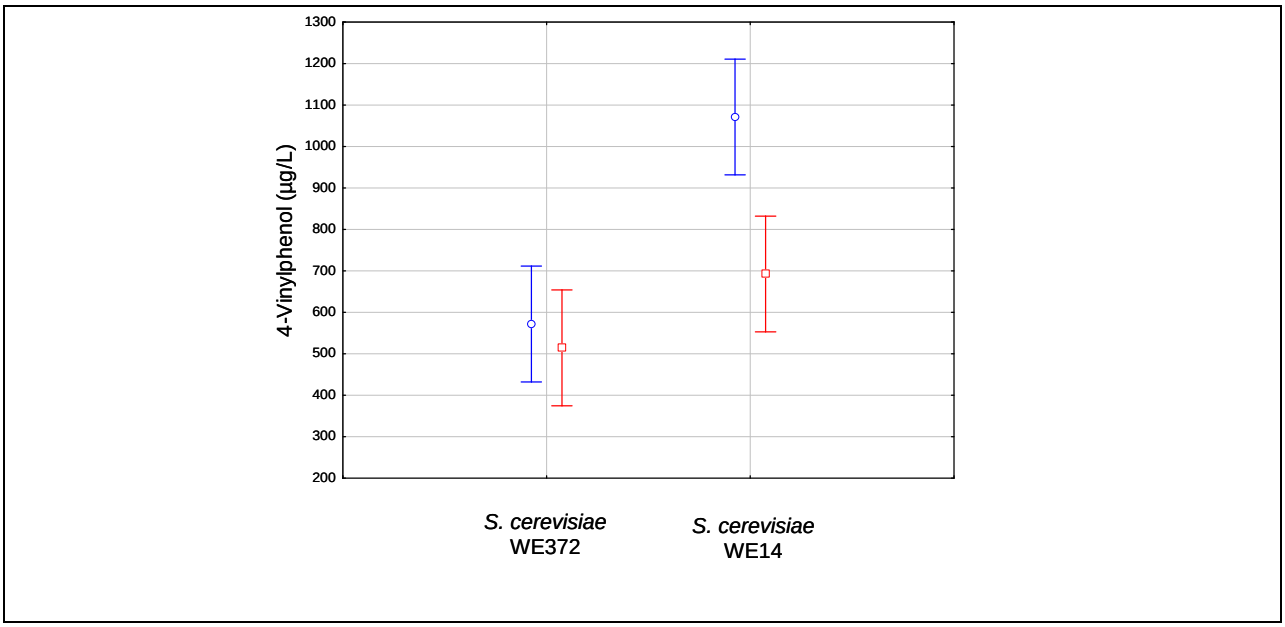

FIGURE 3.17
4-Vinylphenol production in Cabernet Sauvignon 2006 after malolactic fermentation with yeast lees (—) or without yeast lees (—). Alcoholic fermentation performed by the different commercial *S. cerevisiae* yeast strains. Vertical bars denote a 0.95 confidence interval obtained for 4-VP of duplicate treatments.

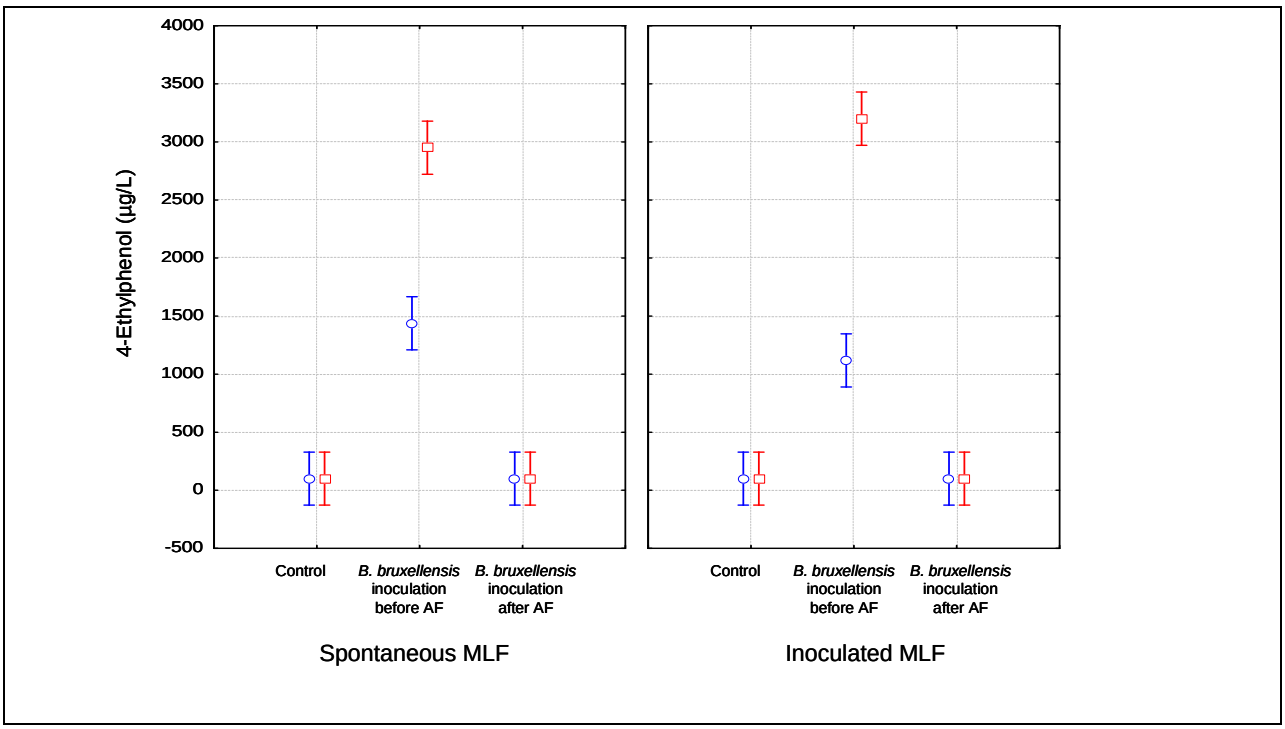

FIGURE 3.18
4-Ethylphenol production in Cabernet Sauvignon 2006 after spontaneous and inoculated malolactic fermentation with yeast lees in control treatments and treatments inoculated with *B. bruxellensis* before or after alcoholic fermentation. Alcoholic fermentation performed by *S. cerevisiae* WE372 (—) or *S. cerevisiae* WE14 (—) strains. Vertical bars denote a 0.95 confidence interval obtained for 4-EP of duplicate treatments.

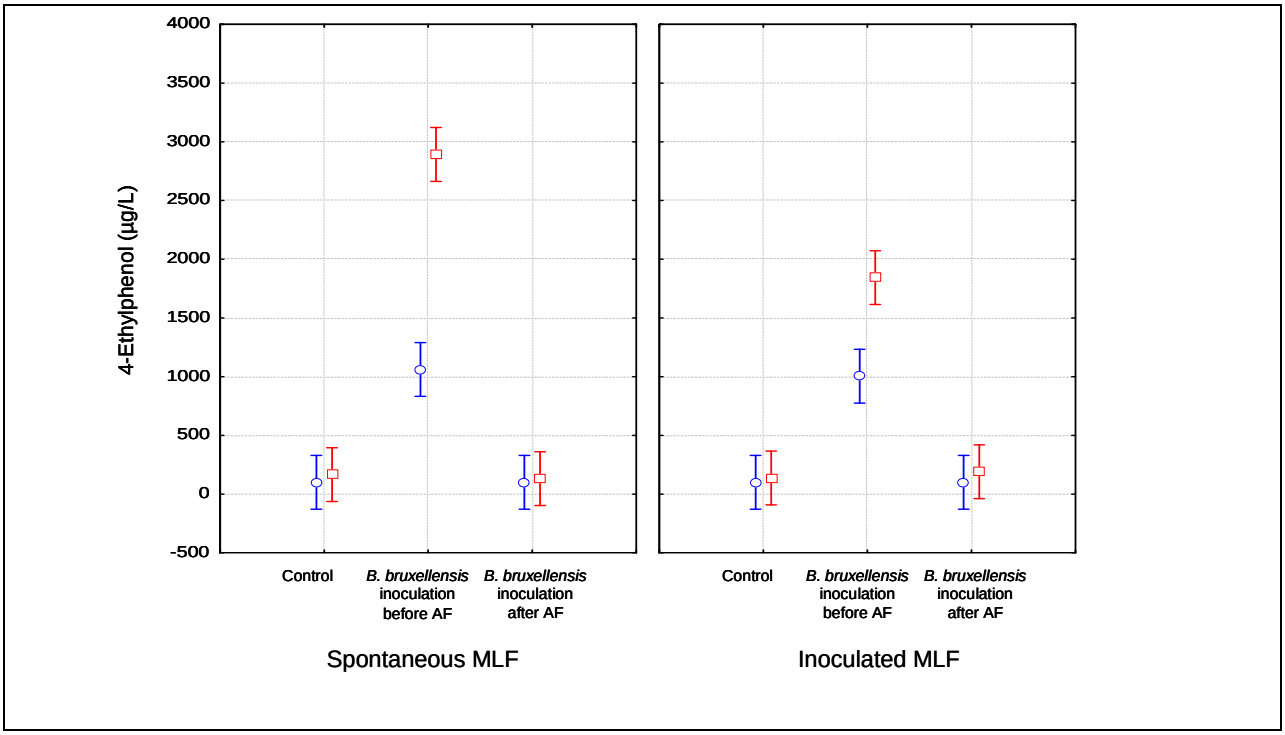

FIGURE 3.19
4-Ethylphenol production in Cabernet Sauvignon 2006 after spontaneous and inoculated malolactic fermentation without yeast lees in control treatments and treatments inoculated with *B. bruxellensis* before or after alcoholic fermentation. Alcoholic fermentation performed by *S. cerevisiae* WE372 (—) or *S. cerevisiae* WE14 (—) strains. Vertical bars denote a 0.95 confidence interval obtained for 4-EP of duplicate treatments.

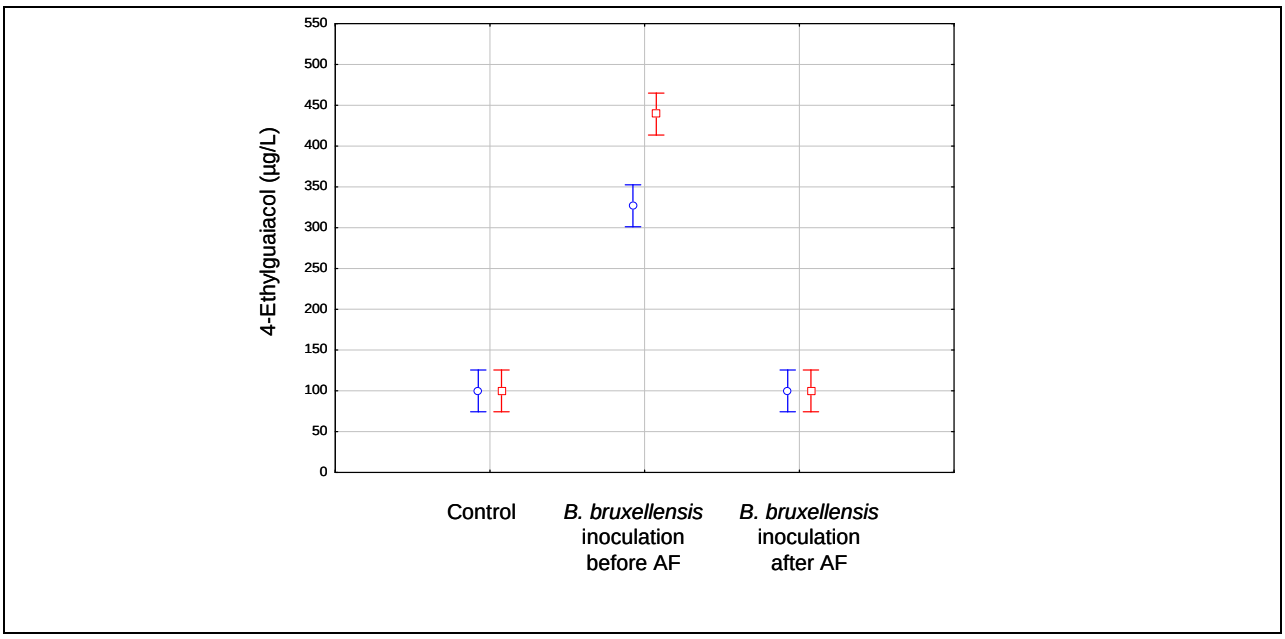

FIGURE 3.20
4-Ethylguaiacol production in Cabernet Sauvignon 2006 after malolactic fermentation in control treatments and treatments inoculated with *B. bruxellensis* before or after alcoholic fermentation. Alcoholic fermentation performed by *S. cerevisiae* WE372 (—) or *S. cerevisiae* WE14 (—) strains. Vertical bars denote a 0.95 confidence interval obtained for 4-EG of duplicate treatments.

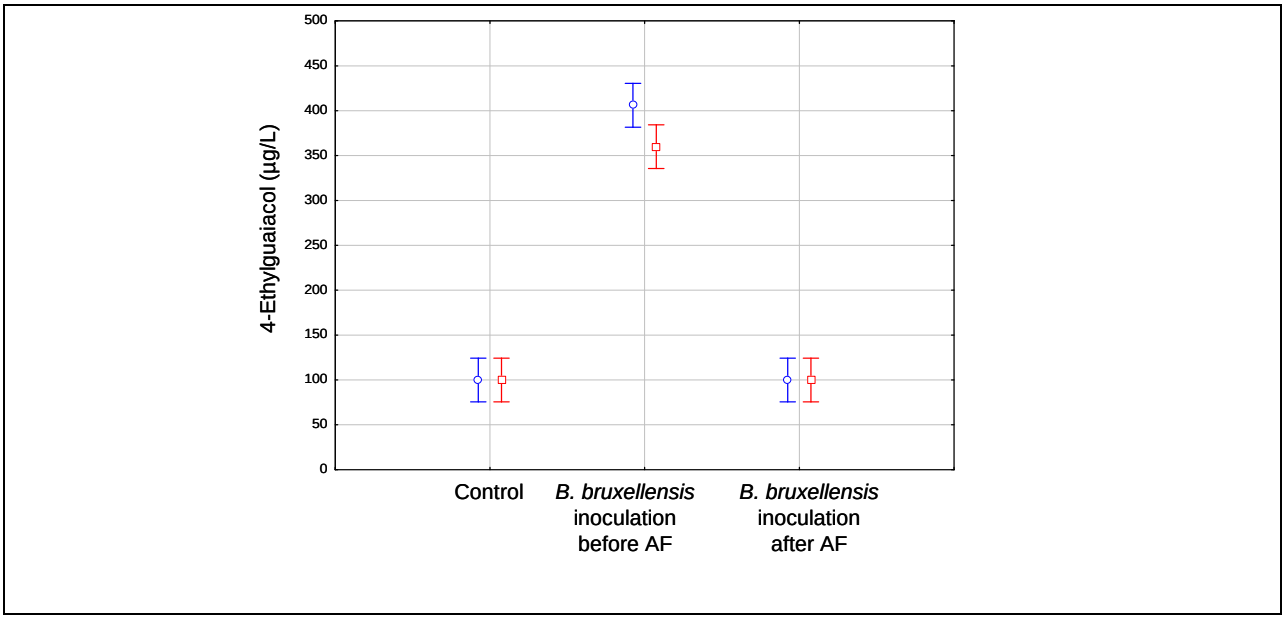

FIGURE 3.21
4-Ethylguaiacol production in Cabernet Sauvignon 2006 after spontaneous (—) and inoculated (—) malolactic fermentation in control treatments and treatments inoculated with *B. bruxellensis* before or after alcoholic fermentation. Vertical bars denote a 0.95 confidence interval obtained for 4-EG of duplicate treatments for both yeast strains.

3.3.2.2 Pinotage 2006

Microbiological analysis of Pinotage 2006 wines after MLF for *Brettanomyces* population showed the same tendency than the Cabernet Sauvignon 2006 wines (data not shown). Higher levels of the total culturable *Brettanomyces* population were found in

wines after inoculated MLF with and without yeast lees. During MLF of Pinotage 2006 the survival of the *Brettanomyces* populations in the treatments inoculated with *B. bruxellensis* B16 after alcoholic fermentation was higher compared to the survival during MLF of the Cabernet Sauvignon 2006 wine. The wines initially fermented with *S. cerevisiae* WE14 also had higher levels of culturable *Brettanomyces* populations after MLF compared to the *S. cerevisiae* WE372 strain.

The levels of 4-VP found after MLF in Pinotage 2006 wines showed a tendency for higher levels in the treatments inoculated with *B. bruxellensis* before alcoholic fermentation regardless the commercial *S. cerevisiae* yeast strain initially used for fermentation or the MLF treatment performed (data not shown). Significantly higher levels of 4-VP could be detected in spontaneous MLF treatments ($p<0.01$) regardless the inoculation stage of *B. bruxellensis* B16, the commercial *S. cerevisiae* yeast strain initially used for fermentation and MLF with or without yeast lees (**Figure 3.22**). The levels of 4-VG were also significantly higher in spontaneous MLF ($p<0.01$) regardless the other treatments performed (**Figure 3.23**). After MLF no significant differences were obtained in the levels of 4-EP found during spontaneous or inoculated MLF. Significant higher levels were found in treatments inoculated with *B. bruxellensis* B16 after alcoholic fermentation compared to inoculation before alcoholic fermentation ($p<0.01$) regardless the commercial *S. cerevisiae* yeast strain initially used for fermentation and the different MLF treatments performed (data not shown). The levels of 4-EP in wines after MLF was also higher in the *S. cerevisiae* WE14 fermented wines and MLF treatments without yeast lees ($p<0.01$) regardless the other treatments performed. **Figure 3.24** illustrates the combined effect of the different treatments on the 4-EP levels in wine after MLF. Significantly higher levels of 4-EP were found in treatments inoculated with *B. bruxellensis* B16 after alcoholic fermentation in wine initially fermented with *S. cerevisiae* WE14 and MLF (regardless spontaneous or inoculated) performed without yeast lees. There was a tendency for higher levels of 4-EG found after spontaneous MLF without yeast lees in wines initially fermented with *S. cerevisiae* WE14 ($p<0.05$), but no significant differences were obtained between the stages of *B. bruxellensis* B16 inoculation (data not shown).

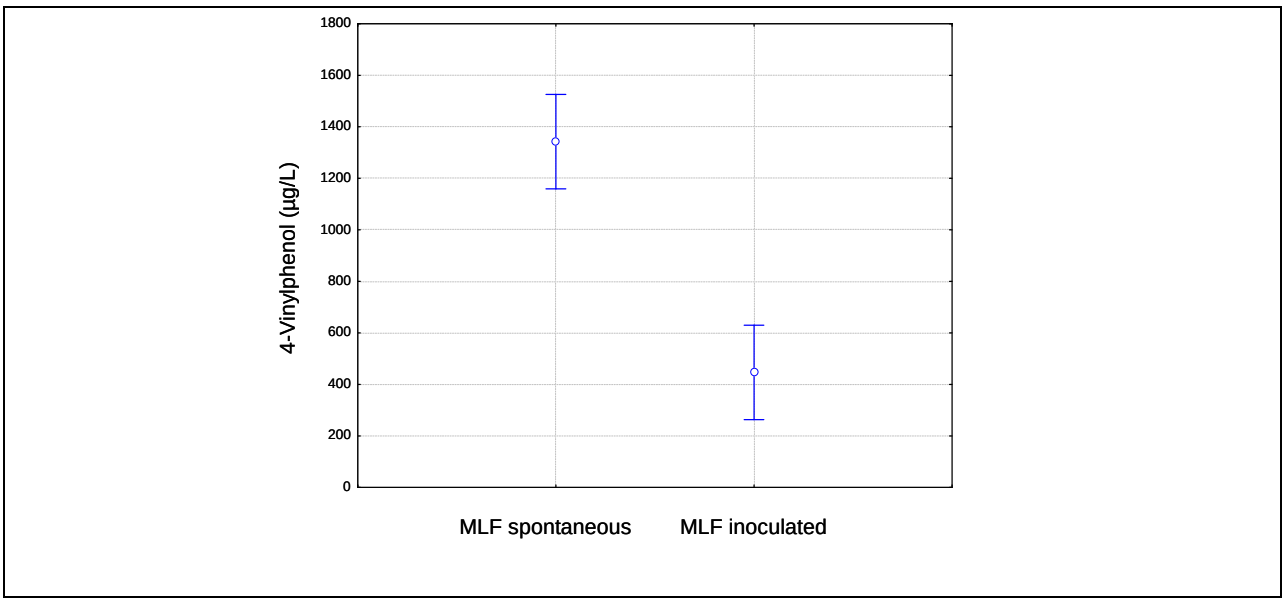

FIGURE 3.22
4-Vinylphenol production in Pinotage 2006 after spontaneous and inoculated malolactic fermentation. Vertical bars denote a 0.95 confidence interval obtained for 4-VP of duplicate treatments for both yeast strains.

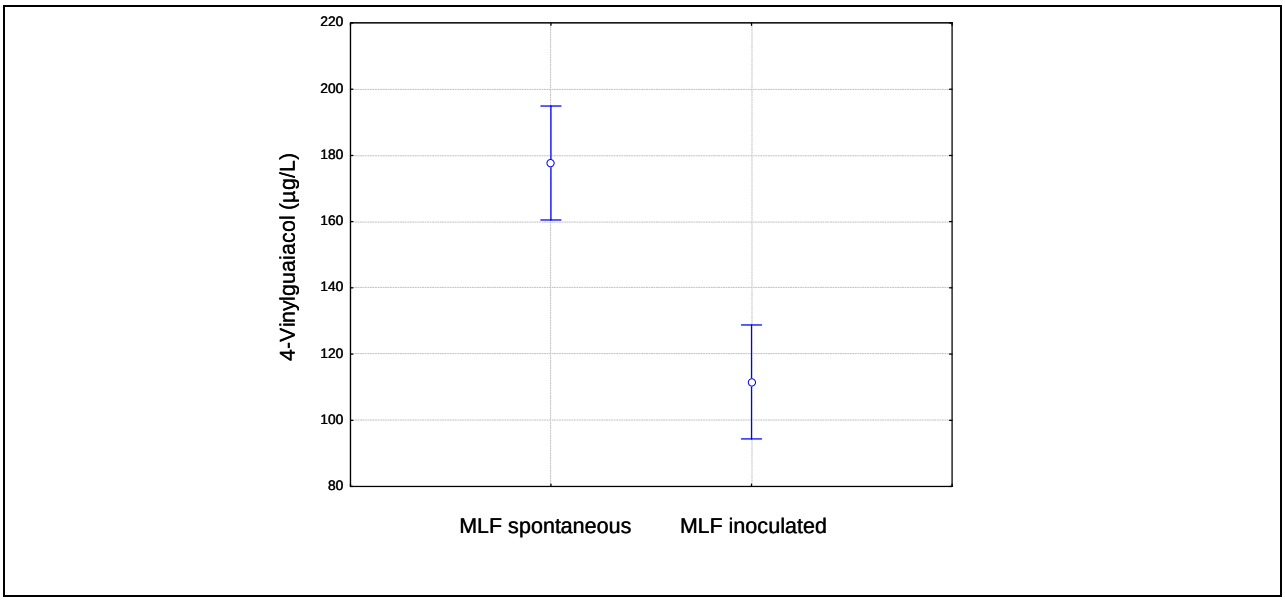

FIGURE 3.23
4-Vinylguaiacol production in Pinotage 2006 after spontaneous and inoculated malolactic fermentation. Vertical bars denote a 0.95 confidence interval obtained for 4-VG of duplicate treatments for both yeast strains.

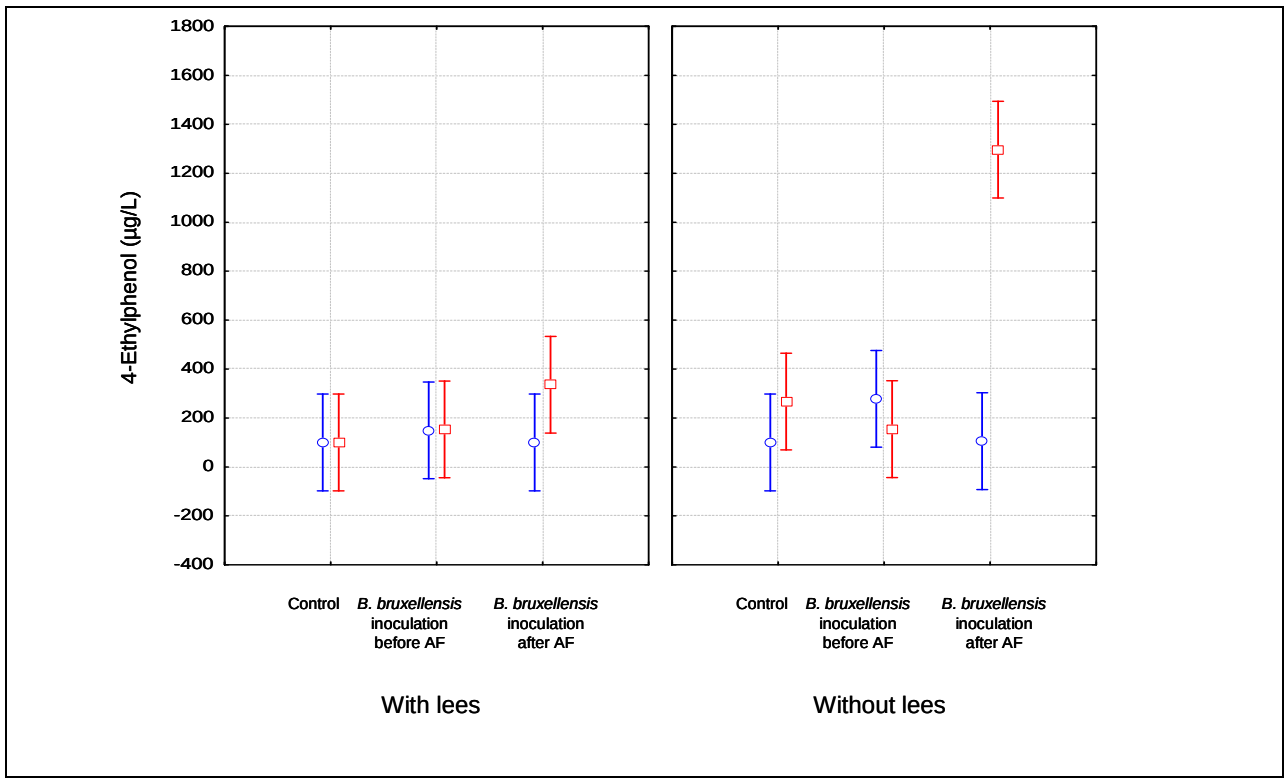

FIGURE 3.24
4-Ethylphenol production in Pinotage 2006 after malolactic fermentation with or without yeast lees in control treatments and treatments inoculated with *B. bruxellensis* before or after alcoholic fermentation. Alcoholic fermentation performed by *S. cerevisiae* WE372 (—) or *S. cerevisiae* WE14 (—) strains. Vertical bars denote a 0.95 confidence interval obtained for 4-EP of duplicate treatments.

3.3.2.3 Cabernet Sauvignon 2007

Microbiological analysis of Cabernet Sauvignon 2007 after MLF showed that the total culturable *Brettanomyces* population was higher in the treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. No *Brettanomyces* populations were detected in the control treatments and very low populations could be detected in treatments inoculated with *B. bruxellensis* B16 after alcoholic fermentation. The results show the same tendency than the Cabernet Sauvignon 2006 and higher culturable *Brettanomyces* populations could also be detected in inoculated MLF treatments.

The levels of 4-VP found in wines after MLF was not significantly influenced by spontaneous or inoculated MLF with or without yeast lees. There is a tendency for the control wine initially fermented with *S. cerevisiae* WE14 to have higher levels of 4-VP ($p<0.05$) (**Figure 3.28**). Overall the wines initially fermented with *S. cerevisiae* WE14 obtained higher levels of 4-VP ($p<0.01$) after MLF regardless the stage of *B. bruxellensis* B16 or the MLF treatments performed (data not shown). Significantly higher levels of 4-VG were also found after MLF in wines initially fermented with *S. cerevisiae* WE14 and very low levels in the *S. cerevisiae* WE372 wines (**Figure 3.27**). There was a tendency for higher levels of 4-VG in control treatments and treatments inoculated with *B. bruxellensis* B16 after alcoholic fermentation. No significant differences could be obtained in the levels of 4-EP after different MLF treatments and wines initially fermented with different commercial *S. cerevisiae* yeast

strains (data not shown). Significantly higher levels of 4-EP were found in treatments inoculated with *B. bruxellensis* before alcoholic fermentation regardless the other treatments performed (**Figure 3.28**). The levels of 4-EG showed exactly the same tendencies than the 4-EP levels found in Cabernet Sauvignon 2007 after MLF (data not shown).

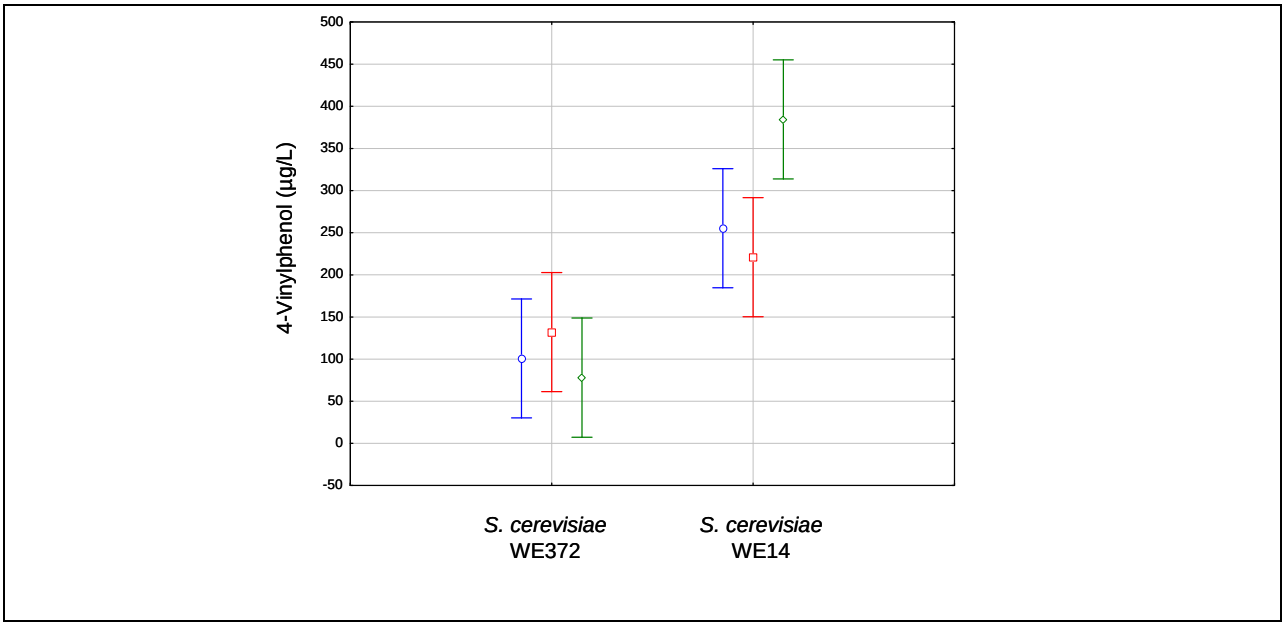

FIGURE 3.26
4-Vinylphenol production in Cabernet Sauvignon 2007 after malolactic fermentation in control treatments (—) and treatments inoculated with *B. bruxellensis* before (—) or after alcoholic (—) fermentation. Alcoholic fermentation performed by the different commercial *S. cerevisiae* yeast strains. Vertical bars denote a 0.95 confidence interval obtained for 4-VP of triplicate treatments.

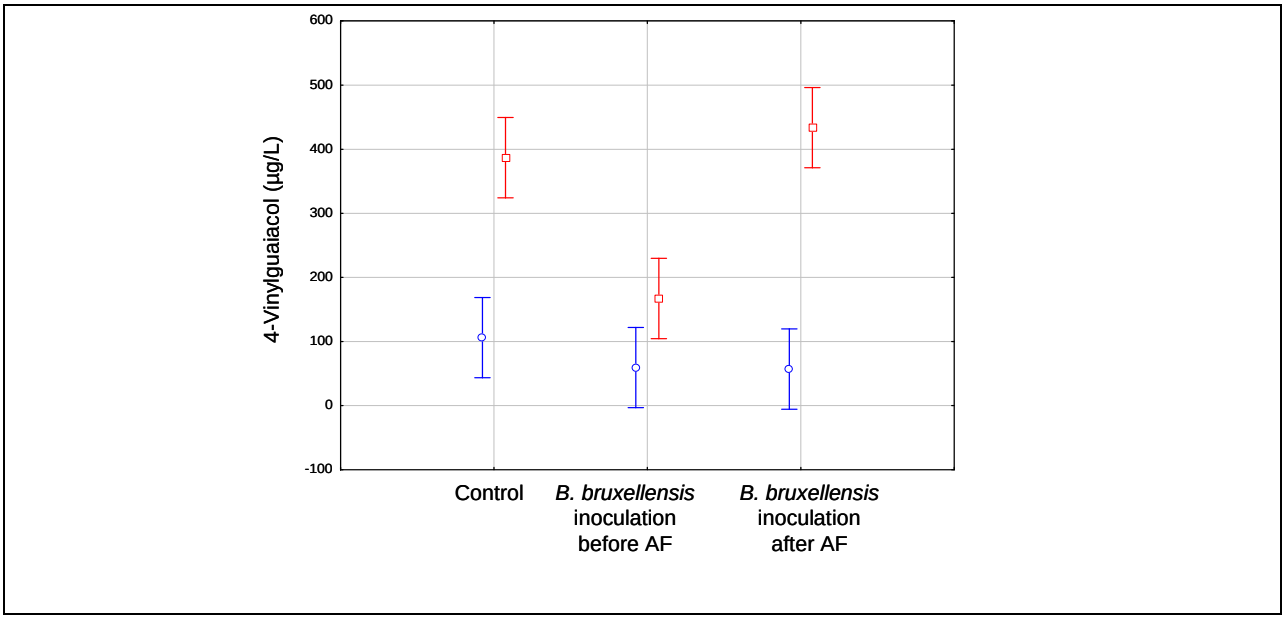

FIGURE 3.27
4-Vinylguaiacol production in Cabernet Sauvignon 2007 after malolactic fermentation in control treatments and treatments inoculated with *B. bruxellensis* before or after alcoholic fermentation. Alcoholic fermentation performed by *S. cerevisiae* WE372 (—) or *S. cerevisiae* WE14 (—) strains. Vertical bars denote a 0.95 confidence interval obtained for 4-VG of triplicate treatments.

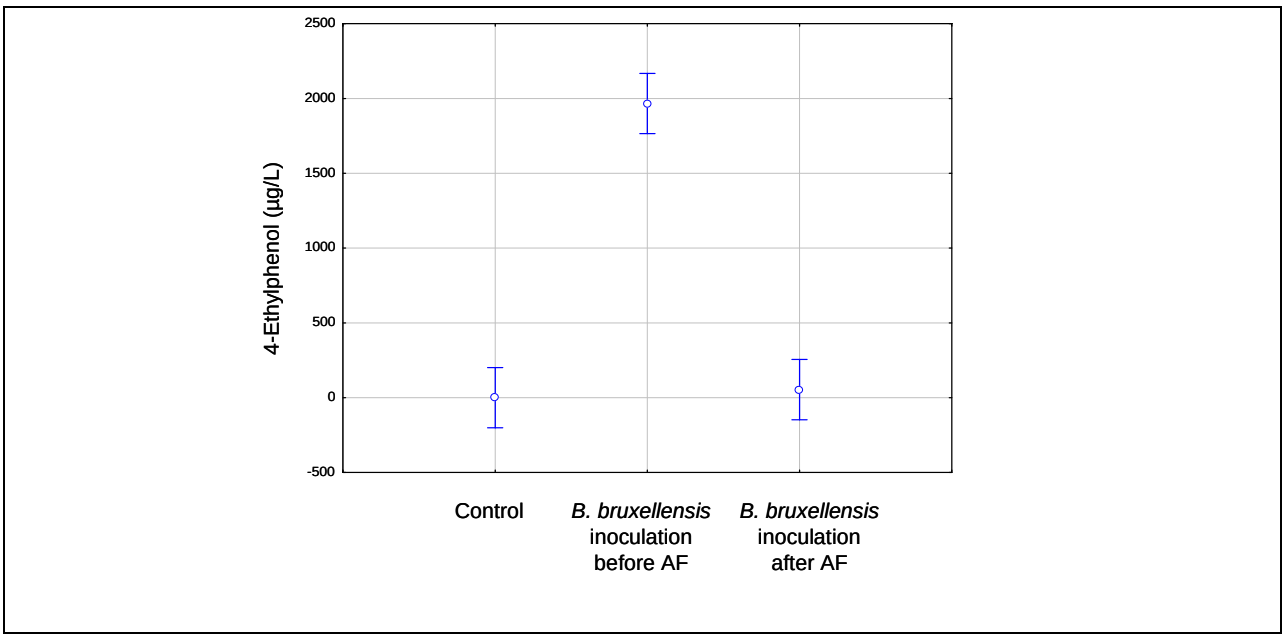

FIGURE 3.28
4-Ethylphenol production in Cabernet Sauvignon 2007 after malolactic fermentation in control treatments and treatments inoculated with *B. bruxellensis* before or after alcoholic fermentation. Vertical bars denote a 0.95 confidence interval obtained for 4-EP of triplicate treatments for both yeast strains.

3.3.2.4 Pinotage 2007

Microbiological analysis of Pinotage 2006 wines after MLF for *Brettanomyces* populations showed the same tendency than the Cabernet Sauvignon 2006 wines (data not shown). Higher levels of the total culturable *Brettanomyces* populations were also found after inoculated MLF with or without yeast lees treatments. There is also a tendency for higher populations in wines initially fermented with *S. cerevisiae* WE14.

No significant differences could be obtained in the levels of 4-VP found in wines after different MLF treatments and stages of *B. bruxellensis* B16 inoculation. There were significantly higher levels of 4-VP after MLF in wines initially fermented with *S. cerevisiae* WE14 regardless all the other treatments performed (data not shown). The same was found for the levels of 4-VG after MLF in wines initially fermented with *S. cerevisiae* WE14 (**Figure 3.29**). After MLF significantly higher levels of 4-EP were found in wines initially fermented with *S. cerevisiae* WE14 (**Figure 3.30**). High levels of 4-EP was found in wine after MLF in both the treatments inoculated with *B. bruxellensis* B16 before and after alcoholic fermentation. No 4-EP was produced in the control treatments. In the MLF treatments without yeast lees there were significantly higher levels of 4-EP ($p<0.01$) regardless the other treatments performed (data not shown). **Figure 3.30** indicates that the highest level 4-EP was obtained in wines initially fermented with *S. cerevisiae* WE14 and inoculated with *B. bruxellensis* B16 after alcoholic fermentation together with performing MLF without yeast lees. The levels of 4-EG found after MLF during the different treatments preformed was the same as for 4-EP. Here there was a tendency for spontaneous MLF wines to have higher levels regardless the other treatments performed (data not shown).

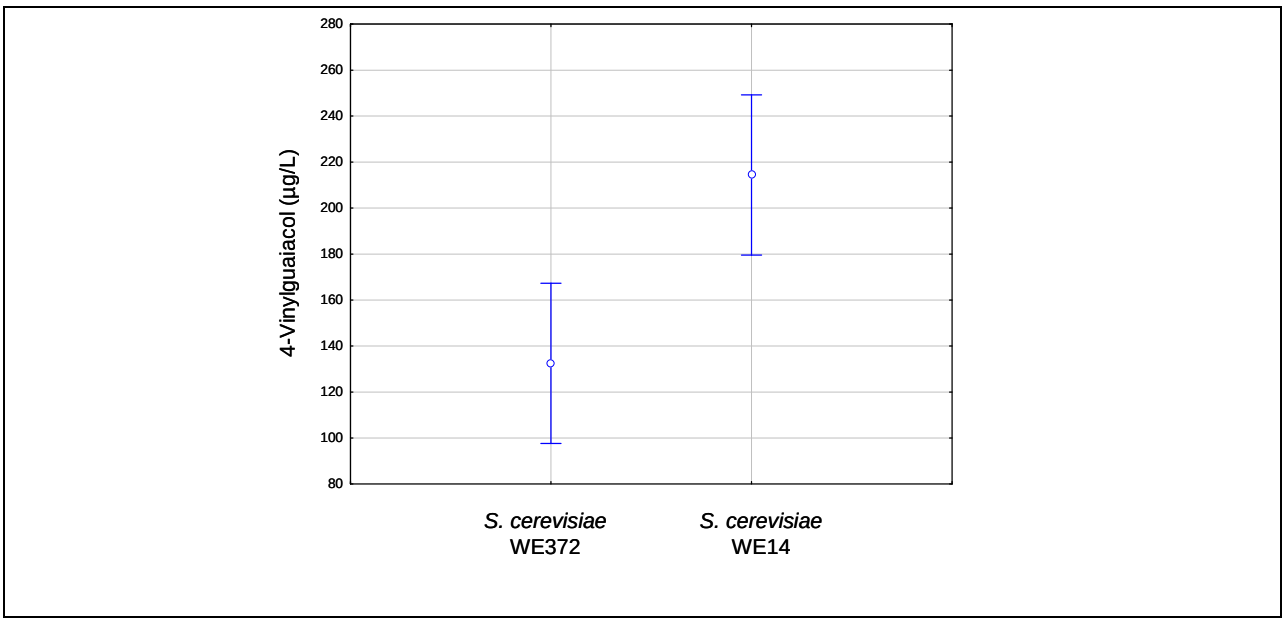

FIGURE 3.29
4-Vinylguaiacol production in Pinotage 2007 after malolactic fermentation. Alcoholic fermentation performed by the different commercial *S. cerevisiae* yeast strains. Vertical bars denote a 0.95 confidence interval obtained for 4-VG of triplicate treatments.

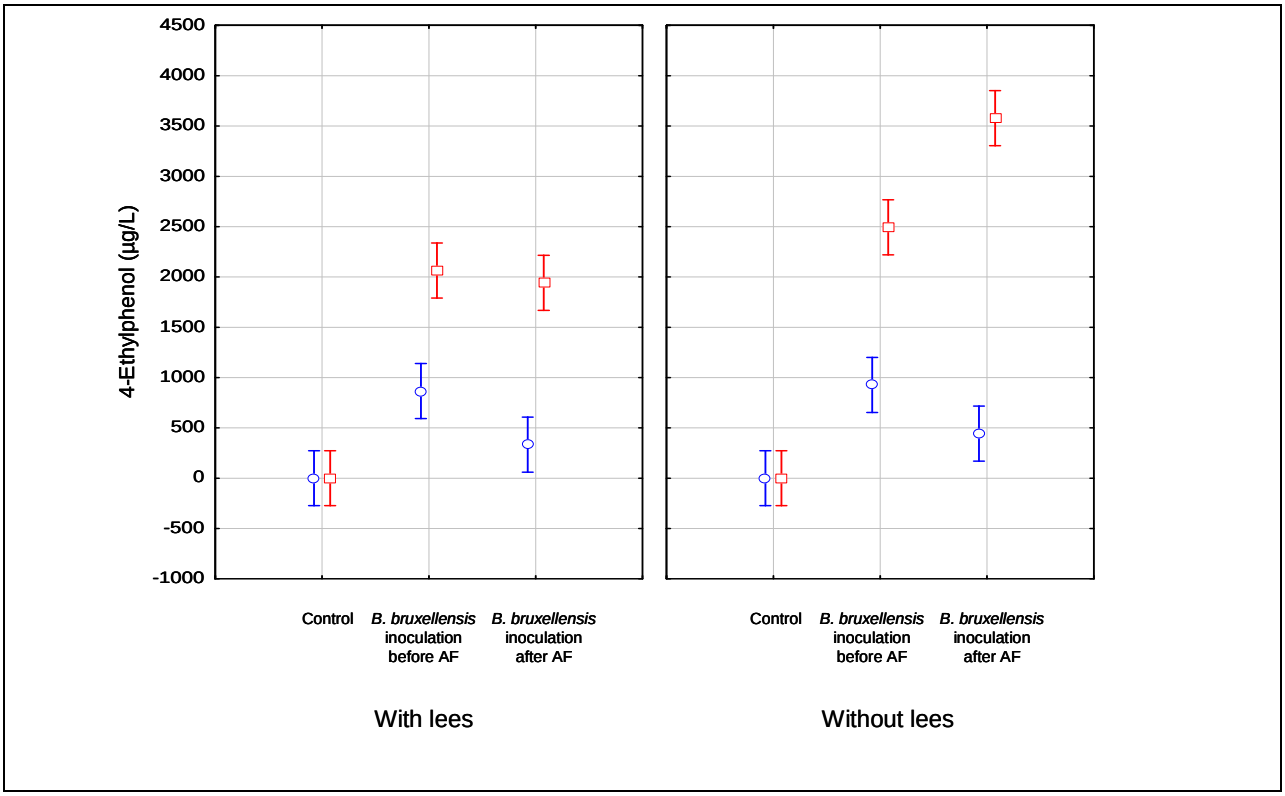

FIGURE 3.30
4-Ethylphenol production in Pinotage 2007 after malolactic fermentation with or without yeast lees in control treatments and treatments inoculated with *B. bruxellensis* before or after alcoholic fermentation. Alcoholic fermentation performed by *S. cerevisiae* WE372 (—) or *S. cerevisiae* WE14 (—) strains. Vertical bars denote a 0.95 confidence interval obtained for 4-EP of triplicate treatments.

3.4 DISCUSSION

The production of volatile phenols by *Brettanomyces* yeast is a major problem in the wine industry and more information is needed to establish ways of reducing the risk of spoilage by these yeasts. To prevent or minimise the effect of *Brettanomyces* yeast on the quality of wine it is important to know during which stages of the winemaking process spoilage could occur and the influence of different winemaking practices on the spoilage capacity of these yeasts. The present study focussed on the production of volatile phenols (4-VP, 4-VG, 4-EP and 4-EG) during different stages of winemaking, the influence of commercial yeast strains and different MLF treatments. The commercial yeast strains *S. cerevisiae* WE372 and *S. cerevisiae* WE14 together with wine isolate *B. bruxellensis* B16 was used in our investigation.

The fermentation performance of the different treatments was monitored to investigate the possible contribution of *B. bruxellensis* inoculation during alcoholic fermentation of different grape juices. For both the varietals and vintages studied it was clear that the *S cerevisiae* WE372 and WE14 fermentations co-inoculated with *B. bruxellensis* initially exhibited a faster fermentation rate. This indicates the contribution of *B. bruxellensis* to the conversion of grape sugars to alcohol. Although *B. bruxellensis* contributed to the initial fermentation rate all the fermentations completed at a similar time and there was no major differences in the ethanol concentrations of the different treatments. Our results correspond to the results found by Renouf *et al*. (2006), except that they found that the co-inoculation of *S. cerevisiae* and *B. bruxellensis* yeast reduces the time necessary to complete alcoholic fermentation. *Brettanomyces* yeast alone will also complete an alcoholic fermentation of grape juice at a much slower rate than *S. cerevisiae* (Boulton *et al*., 1996).

With the enumeration of *Brettanomyces* it is important to note that the yeasts can survive in a viable but non-culturable state in wine stimulated by undesirable conditions such as oxygen deficiency and sulphur dioxide additions (Millet & Lonvaud-Funel, 2000; Du Toit *et al*., 2005). This makes the enumeration of *Brettanomyces* populations with selective agar plates difficult with a possibility of underestimating the population densities present in wines.

The potential growth and survival of *B. bruxellensis* during alcoholic fermentation in different grape varieties and its interaction with other wine yeasts has not before been investigated in detail. Microbiological analysis showed that although there was a reduction in the population from the initial inoculation density *B. bruxellensis* B16 could survive during alcoholic fermentation in both varietals and vintages studied. A study by Dias *et al*. (2003b) has also shown that *Brettanomyces* yeast populations can survive during alcoholic fermentation even if the initial inoculum size was as low as 10 cells/mL. It has also been stated that the production of 4-EP is not subjected to the catabolic repression by glucose and that 4-EP can be produced during alcoholic fermentation (Dias *et al*., 2003b). Therefore wine contamination by *Brettanomyces* yeast from the onset of alcoholic fermentation can lead to the production of wine with elevated levels of 4-EP (Rodrigues *et al*., 2001). The reduction in the inoculation density during the

Pinotage 2006 fermentation was lower compared to the Cabernet Sauvignon 2006. Therefore the culturability of *Brettanomyces* populations in Pinotage wines seems to be higher. A possible explanation could be that less inhibitory compounds is present in these wines and therefore better survival of *B. bruxellensis* B16 in these wines. Overall, much higher *Brettanomyces* populations could be detected in the 2007 compared to the 2006 vintage in both varietals studied. Factors such as the origin of the grapes, environmental condition during the ripening season of the grapes, and the health status of the grapes could lead to variation in the grape must composition and possibly lead to the variation in *B. bruxellensis* cell numbers obtained during this experiment. The occurrence of rain and rot during the 2006 season could also have influenced the natural microflora found on the grapes. The natural microflora found (mostly *Hanseniaspora*/*Kloeckera* spp., *Saccharomyces* spp. and *Rhodotorula* spp.) was higher on the 2006 grapes (approximately 10^3 cells/mL) (data not shown) indicating a better survival of natural flora on these grapes that could have exerted an inhibitory effect on the growth of *B. bruxellensis* later in the wine. A study by Comitini *et al*. (2004) described the production of killer toxins by *Pichia anomala* and *Kluyveromyces wickerhamii* that exerts a fungicidal activity against *Brettanomyces* yeasts. Although those specific natural yeasts were not detected by simple plating on media during this study it does not exclude their possible contribution towards the effect on *B. bruxellensis* growth. There are numerous other factors that could also affect the growth of *B. bruxellensis* including the ethanol concentration of the wine (Dias *et al*., 2003b; Medewar *et al*., 2003; Rodrigues *et al*., 2001; Suárez *et al*., 2007). Medewar *et al*. (2003) suggested a critical ethanol concentration of 11.5 %(v/v) in an alcoholic media above which *Brettanomyces* yeast cells cease to reproduce and a decrease in cell numbers was observed. Contrary to these results Dias *et al*. (2003) found that *Brettanomyces* cell numbers could be found at ethanol concentrations of 12 %(v/v), but no growth was found at 13 %(v/v) in synthetic wine media. During our study most of the wines had ethanol concentrations higher than 13 %(v/v) indicating that *B. bruxellensis* can survive at very high ethanol concentrations in red wine. It was also clear that the alcoholic fermentations performed by *S. cerevisiae* WE372 allowed higher detection of culturable populations of *Brettanomyces* compared to the *S. cerevisiae* WE14 fermentations for both varietals and vintages studied. It is well known that during fermentation a complex mixture of yeasts exists and interact with one another (Renouf *et al*., 2006). Both the *S. cerevisiae* strains tested are killer positive yeasts with the potential to dominate the fermentation and could therefore possibly restrict the growth of *B. bruxellensis*. The WE14 fermentations showed lower culturable *Brettanomyces* populations indicating that the WE14 yeasts exerted an inhibitory effect on the growth of *B. bruxellensis* B16. The total non-*Saccharomyces* population in the WE14 fermentations were also lower when compared to the WE372 fermentations suggesting that this *S. cerevisiae* strain influenced the other yeasts.

The enumeration of *Brettanomyces* populations after MLF indicated that the highest culturable populations could be detected in the treatments inoculated with

B. bruxellensis B16 before alcoholic fermentation. This indicates that better survival of *B. bruxellensis* B16 during MLF for treatments inoculated before alcoholic fermentation compared to treatments inoculated after alcoholic fermentation. The growth of *Brettanomyces* is stimulated under conditions that allow moderate aeration (Aguilar Uscanga *et al*., 2003; Boulton *et al*., 1996). During winemaking certain techniques such as the transfer of wines after alcoholic fermentation, malolactic fermentation practises and wine aging could allow the wines to come in contact with oxygen. These conditions could lead to the stimulation of *Brettanomyces* yeast growth and therefore the period after alcoholic fermentation is considered the most dangerous stage during winemaking for contamination to occur (Rodrigues *et al*., 2001). This suggested that if contamination occurred after alcoholic fermentation higher *B. bruxellensis* cell numbers would be present after MLF. Contrary to this statement, we found during this study that if *B. bruxellensis* inoculation occurred before the onset of alcoholic fermentation these yeast would have longer time to adapt to the wine conditions (for example the increase in ethanol during alcoholic fermentation) and reach higher population densities during later stages of winemaking. Comparing the different MLF treatments there was a tendency for higher culturable populations of *Brettanomyces* spp. found in inoculated MLF wines.

Brettanomyces yeasts have been recognised to produce high levels of acetic acid (Boulton *et al*., 1996; Ciani & Ferraro, 1997). High levels of acetic acid are undesirable, leading to an increase in the volatile acidity associated with negative sensory characteristics of wine (Boulton *et al*., 1996; Arvik & Henick-Kling, 2002). Levels above 0.8 g/L become objectionable leading to a development of a vinegar character in wine (Swiegers *et al*., 2005). During all the seasons and with all the grape varieties studied the wines inoculated with *B. bruxellensis* B16 produced slightly higher volatile acidity levels after alcoholic fermentation. There was also a tendency for higher volatile acidity levels in wines fermented with *S. cerevisiae* WE372. Higher volatile acidity levels were also obtained after MLF in wines inoculated with *B. bruxellensis* before and after alcoholic fermentation (between 0.5 g/L and 0.8g/L) compared to the control treatments. Acetic acid bacteria can also produce acetic acid and lead to high VA levels in wines (Du Toit & Lambrechts, 2002). The possible contribution of acetic acid bacteria to the VA levels during this study is very limited, because no acetic acid bacteria were detected after alcoholic fermentation and MLF in these wines.

It is important to note during this discussion that the higher levels of volatile phenols found in the 2007 wines compared to the 2006 wines is due to the fact that 10 mg/L of *ρ*-coumaric and 1 mg/L of ferulic acid were added to the juice before the onset of alcoholic fermentation in 2007.

The production of volatile phenols by *Brettanomyces* yeasts during alcoholic fermentation in different grape varietals fermented with different commercial *S. cerevisiae* yeast strains is not well researched. The production of 4-VP and 4-VG was significantly higher after alcoholic fermentation in the Cabernet Sauvignon 2006 wine for the control treatments fermented with *S. cerevisiae* WE14 yeast strain. In the

Pinotage 2006 wine higher levels of 4-VP were found after alcoholic fermentation by *S. cerevisiae* WE14 in treatments inoculated with *B. bruxellensis* before alcoholic fermentation. No significant differences were found in the production of 4-VG between the different commercial *S. cerevisiae* yeast strains used for alcoholic fermentation and control treatments or treatments inoculated with *B. bruxellensis* B16. No significant differences in the levels of 4-VP and 4-VG were obtained in the Cabernet Sauvignon 2007 wine fermented with the different commercial *S. cerevisiae* yeast strains. There were tendencies of higher levels of these two compounds in the control treatments compared to the treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. The production of 4-VP and 4-VG was significantly higher after alcoholic fermentation in the Pinotage 2007 wine fermented with the *S. cerevisiae* WE14 yeast strain compared to the wines fermented with *S. cerevisiae* WE372 yeast strain. This part of our study clearly indicates the contribution of commercial *S. cerevisiae* yeast strains on the production of 4-VP and 4-VG during alcoholic fermentation with higher levels usually found in wines fermented with the *S. cerevisiae* WE14 yeast strain. Chatonnet *et al*. (1993) studied the synthesis of volatile phenols by *S. cerevisiae* yeasts during the production of wine. They found that *S. cerevisiae* yeasts were able to transform both *ρ*-coumaric and ferulic acid to 4-VP and 4-VG, respectively, and so established the decarboxylase activity of these yeasts. They also stated that this activity only occurs during alcoholic fermentation and no vinylphenol can be synthesised in dry wines by these yeasts. A study by Van Wyk & Rogers (2000) investigated the potential of different *S. cerevisiae* wine yeast strains to produce 4-VG during the fermentation of Kerner wines. They found significant differences in the production of 4-VG between the different wine yeast strains and classified the WE372 strain as a high and the WE14 strain as a low producer. This does not correlate with our results that indicated that the WE14 yeast strain produce higher levels of the vinyl derivatives.

The production of 4-EP and 4-EG was significantly higher in Cabernet Sauvignon 2006, 2007 and Pinotage 2007 wines after alcoholic fermentation for the treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation. The levels produced of these two compounds were much higher than the aroma thresholds in red wines of 600 µg/L and 110 µg/L for 4-EP and 4-EG, respectively (Chatonnet *et al*., 1995). This clearly indicates that the levels of these compounds produced during alcoholic fermentation will definitely lead to a depreciation of wine aroma. Much higher levels of ethyl derivatives (approximately double) were found in the 2007 wines compared to the 2006 wines clearly indicating that the additional supplementation of hydroxycinnamic acids enhanced the production of 4-EP and 4-EG. No 4-EP and 4-EG was produced in the control treatments and during the fermentation of Pinotage 2006 wines. No significant differences in the levels of ethyl derivatives could be obtained after alcoholic fermentation in the Cabernet Sauvignon 2006 and 2007 wines fermented with *S. cerevisiae* WE372 or *S. cerevisiae* WE14. Significantly higher levels of 4-EP and 4-EG was produced after alcoholic fermentation in the Pinotage 2007 wines fermented with *S. cerevisiae* WE372 compared to the *S. cerevisiae* WE14 yeast strain.

Overall there is a tendency for the Cabernet Sauvignon varietal to have higher levels of ethyl derivatives after alcoholic fermentation compared to the Pinotage varietal during both vintages investigated. The very high level of ethanol found in the Pinotage wines could have had an inhibitory effect on the production of volatile phenols by *B. bruxellensis* B16 (**Table 3.5**). Dias *et al*. (2003b) found no *Brettanomyces* yeast growth and production of 4-EP at ethanol levels above 13 %(v/v) in synthetic media. In another study by Rodrigues *et al*. (2001) they found that very high levels of ethanol [higher than 13 %(v/v)] yielded low levels of 4-EP. Another explanation could be that higher hydroxycinnamic acid precursors are naturally present in the Cabernet Sauvignon grape juice. It is well known that the total amount of volatile phenols found in wines depends on the amount of precursors (*ρ*-coumaric, ferulic and caffeic acids) present in grapes and that these concentrations vary between the different grape varieties (Rodrigues *et al*., 2001; Morel-Salmi *et al*., 2006; Morata *et al*., 2007; Rentzsch *et al*., 2007).

To date there is very limited information available on the production of volatile phenols during different MLF practices. This information could be very valuable to winemakers to aid in better control of wine quality if *Brettanomyces* yeast spoilage is a problem. In the Cabernet Sauvignon wine after MLF no differences were obtained in the levels of 4-VP and 4-VG between the MLF spontaneous and inoculated treatments. Regardless of the other treatments performed, significantly higher levels of 4-VP were found in wines after MLF with yeast lees treatments initially fermented with *S. cerevisiae* WE14. The control treatments also had a tendency to have higher levels of 4-VP. There was also a tendency for wines after MLF initially fermented with *S. cerevisiae* WE14 to have higher levels of 4-VG. After spontaneous MLF significantly higher levels of 4-VP and 4-VG were found in Pinotage 2006 wine, regardless of the other treatments performed. This clearly indicates the contribution of spontaneous MLF towards higher levels of vinyl derivates found in wines after MLF. In the Cabernet Sauvignon 2007 and Pinotage 2006 no significant differences were obtained in the levels of 4-VP and 4-VG between the different MLF treatments. There was a tendency for higher levels of vinyl derivatives in the control treatments after MLF in the Cabernet Sauvignon 2007 wines. Higher levels of the vinyl derivatives were found in wines initially fermented with *S. cerevisiae* WE14 in both the Cabernet Sauvignon 2007 and Pinotage 2007 wines after MLF, regardless of the other treatments performed. This clearly indicates the contribution of the commercial yeast strain towards the levels of vinyl derivatives found in wines after MLF was completed. The only significant differences in the production of 4-EP and 4-EG in Cabernet Sauvignon 2006 wines was that higher levels were obtained in treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation initially fermented with the *S. cerevisiae* WE14 yeast strain. No differences were obtained in the levels of 4-EP between spontaneous and inoculated MLF, but there is a tendency for higher levels in MLF treatments performed with yeast lees. Spontaneous MLF also showed the tendency to have higher 4-EG levels. After MLF of the Pinotage 2006 and Pinotage 2007 wine significantly higher levels of 4-EP and 4-EG was also

produced in wines initially fermented with *S. cerevisiae* WE14, regardless the other treatments performed. The levels of 4-EP and 4-EG was also higher in some of the MLF treatments performed without yeast lees and there was a tendency for higher levels of 4-EG in spontaneous MLF. For both the Pinotage vintages there was a tendency for higher ethyl derivatives to be produced in wines after MLF in treatments inoculated with *B. bruxellensis* B16 after alcoholic fermentation. The levels of the ethyl derivatives was significantly higher after MLF in Cabernet Sauvignon 2007 wine in treatments inoculated with *B. bruxellensis* B16 before alcoholic fermentation.

In general there seems to be a tendency for a higher production of volatile phenols by *B. bruxellensis* in wines initially fermented with the WE14 yeast strain after MLF. Furthermore spontaneous MLF could lead to the production of higher levels of volatile phenols. Cavin *et al*. (1993) found that *p*-coumaric acid and ferulic acid can be decarboxylated by *Lactobacillus brevis, L. plantarum* and *Pediococcus*. Considering that these lactic acid bacteria strains mostly dominate spontaneous MLF higher levels of the vinyl derivatives can be produced and then transformed to the ethyl derivatives by *B. bruxellensis*. Another study indicated the possibility of the reduction step from the vinyl derivatives to the ethyl derivatives in *L. brevis, L. collinoides* and *L. plantarum* (Couto *et al*., 2006). Therefore *Lactobacillus* spp. present during spontaneous MLF could have contributed to the final level of 4-EP and 4-EG produced in the wines. Chatonnet *et al*. (1995) illustrated the ability of *L. plantarum* to produce 4-EP, but concluded that the influence of LAB on the volatile phenol content of wine is very restricted under oenological conditions. There also seems to be a tendency in our study for MLF treatments with yeast lees to exhibit lower levels of volatile phenols. It has been found that yeast cell walls can lead to a loss in aroma compounds of wine (Lubbers *et al*., 1994). Guilloux-Benatier *et al*. (2001) illustrated a decrease in 4-EP and 4-EG levels in red wine aged on yeast lees compared to wine aged without the presence of yeast lees. A study done by Chassagne *et al*. (2005) indicated that yeast lees could lead to a significant reduction in the 4-EP and 4-EG content of wine. They stated that it is possible for yeast lees to adsorb volatile phenols in wine.

3.5 CONCLUSION

Brettanomyces bruxellensis is capable of surviving during alcoholic fermentation and can produce high levels of volatile phenols. The levels of ethyl derivatives produced during alcoholic fermentation can lead to a depreciation of wine quality. Different commercial *S. cerevisiae* wine yeast strains interact differently with *B. bruxellensis* yeast and influence the volatile phenol production in wine during alcoholic fermentation and malolactic fermentation.

Certain MLF practices can also influence the production of volatile phenols during the winemaking process. Spontaneous MLF can in certain cases lead to elevated levels of volatile phenols. Conducting MLF with yeast lees can significantly reduce the levels

of ethyl derivatives in wine and therefore lower the risk of the negative contribution of these compounds to wine aroma.

To avoid the production of wines with elevated levels of volatile phenols it is important to control *Brettanomyces* contamination from the early stages of winemaking, before alcoholic fermentation. It is suggested that low 4-VP and 4-VG producing wine yeast strains should be used to conduct alcoholic fermentation. Furthermore, spontaneous MLF should be avoided if *Brettanomyces* yeast is a problem in a specific winery. The combination of inoculated MLF with yeast lees can aid in a reduction of volatile phenol concentrations of wine.

3.6 ACKNOWLEDGEMENTS

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Chapter 4

RESEARCH RESULTS

The production of volatile phenols by *Saccharomyces cerevisiae* wine yeast strains and lactic acid bacteria associated with wine

4. RESEARCH RESULTS

The production of volatile phenols by *Saccharomyces cerevisiae* wine yeast strains and lactic acid bacteria associated with wine

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Different commercial yeast strains were tested for their ability to produce volatile phenols in red grape juice under winemaking conditions and in synthetic grape juice under laboratory conditions. Results indicated that all the commercial yeast strains could produce 4-vinylphenol and 4-vinylguaiacol from ρ-coumaric acid and ferulic acid respectively during alcoholic fermentation. Differences between the yeast strains in their potential to produce volatile phenols were evident during this study. Higher concentrations of the vinyl-derivatives were produced in synthetic grape juice in comparison to red grape juice. Commercial yeast strains were also screened for the presence of the phenyl acrylic acid decarboxylase gene and all the yeast strains tested positive for the *PAD1* gene. Furthermore, 23 strains of wine lactic acid bacteria were screened for the presence of the phenolic acid decarboxylase (*padA*) gene. The strains that contained the *padA* gene were evaluated for the ability to produce volatile phenols in synthetic wine media. Results showed that 52% of the LAB strains in this study tested positive for *padA*. The production of vinyl-derivatives by lactic acid bacteria in synthetic wine media was very low, except for the production of 4-vinylphenol by *L. plantarum* 78. During this study none of the commercial yeast strains and lactic acid bacteria strains tested could produce 4-ethylphenol and 4-ethylguaiacol.

4.1 INTRODUCTION

The aroma and flavour of wine is commonly derived from the grape, but not all the grape derived flavour is present in the grape before the onset of the winemaking process (Pollnitz *et al*., 2000). The ability of microorganisms to produce flavour compounds from flavourless grape-derived precursors is therefore of great importance to the final quality of the wine.

Yeast starter cultures are commonly used for the onset of and successful completion of alcoholic fermentation of grape juice. The yeast strains selected for starter cultures typically belong to *Saccharomyces cerevisiae* and related *Saccharomyces* species. These yeasts have characteristics that can positively contribute to wine production, including high fermentation rates, successful completion of fermentation, good ethanol resistance, temperature, SO_2 and sugar tolerance, and favourable aroma production (Boulton *et al*., 1996). Although these yeasts can produce a variety of esters

that can contribute to the fruity character of wine they can however also produce other less desirable flavour compounds such as volatile phenols, acetic acid and sulphur-containing compounds (Chatonnet *et al*., 1992; Swiegers *et al*., 2005).

Lactic acid bacteria (LAB) are also part of the natural flora of wine and the genera with the most importance in winemaking include *Lactobacillus*, *Pediococcus*, *Oenococcus* and *Leuconostoc* (Davis *et al.,* 1988; Couto *et al*., 2006). LAB is responsible for malolactic fermentation relating to the conversion of L-malic acid to L-lactic acid in wines. The advantages of malolactic fermentation include the deacidification of wine and increased microbiological stability. In addition to malolactic fermentation, LAB can also produce aroma and flavour compounds that affect the final quality of wine. Previous studies have shown that LAB can also produce volatile phenols (Cavin *et al*., 1993; Chatonnet *et al*., 1995; Couto *et al*., 2006).

Volatile phenols originate from precursors naturally present in grapes known as hydroxycinnamic acids. Cinnamic acids are usually present in grapes esterified with tartaric acid and include caftaric acid, coutaric acid and fertaric acid (Boulton *et* al., 1996; Lao *et al*., 1997). The free form of hydroxycinnamic acids, including *ρ*-coumaric acid, ferulic acid and caffeic acid, can be released by cinnamoyl esterase activities during the winemaking process (Dugelay *et al*., 1993; Stead, 1995). The formation of volatile phenols involves the decarboxylation of hydroxycinnamic acid precursors (*ρ*-coumaric acid ferulic acid) by a hydroxycinnamate decarboxylase enzyme to produce vinyl derivatives (4-vinylphenol and 4-vinylguaiacol) (Edlin *et al*., 1995). The action of vinyl phenol reductase then further reduces the vinyl derivatives to the ethyl derivatives (Dias *et al*., 2003).

The most influential volatile phenols include 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol (Chatonnet *et al*., 1992). In more recent years, the presence of 4-ethylcatechol in wines has also been reported to have an influence on wine aroma (Hesford *et al*., 2004). High concentrations of volatile phenols can cause a phenolic off-flavour, and the general aroma description of wines with high levels of volatile phenols includes phenolic, medicinal, horse sweat, leather, smoky etc. (Chatonnet *et al*., 1992; Rodrigues *et al*., 2001). The contribution of volatile phenols towards wine quality varies, depending on the wine style and the ratio between the concentrations of the different volatile phenols (Chatonnet *et al*., 1993, 1995; Suárez *et al*., 2007). Concentrations higher than 725 µg/L of 4-vinylphenol and 4-vinylguaiacol together could lead to phenolic or pharmaceutical odour characters in wines (Chatonnet *et al*., 1993). The aroma thresholds for 4-ethylphenol and 4-ethylguaiacol range from 440 to 600 µg/L and 33-110 µg/L respectively, depending on the wine style (Chatonnet *et al*., 1995; Curtin *et al*., 2005).

The phenolic off-flavour production by *S. cerevisiae* is controlled by phenyl acrylic acid decarboxylase gene (*PAD1*) that encodes phenyl acrylic acid decarboxylase enzyme responsible for the transformation of hydroxycinnamic acids into the vinyl derivatives (Clausen *et al*., 1994; Shinohara *et al*., 2000). LAB possess an inducible phenolic acid decarboxylase activity (PAD), encoded by the *padA* gene (Cavin *et al*.,

1997; Barthelmebs *et al*., 2000). The expression of the *padA* gene is transcriptionally regulated by the presence of phenolic acids (Cavin *et al*., 1997).

The main objective of this work was to evaluate the ability of different yeast strains to produce volatile phenols during alcoholic fermentation of red grape juice under winemaking conditions. The ability of the commercial *S. cerevisiae* yeast strains to produce volatile phenols was also evaluated in synthetic grape must under laboratory conditions to eliminate the influence of other wine microorganisms. The presence of the *PAD1* gene in the different commercial *S. cerevisiae* yeast strains was evaluated by polymerase chain reaction (PCR) screening. Furthermore, the presence of the *padA* gene in different strains of LAB was also evaluated by PCR screening and the production of volatile phenols by the positive strains was tested in synthetic wine media. The results of this study could possibly indicate the influence of wine microorganisms, other than *Brettanomyces* yeast, on the production of volatile phenols in wine.

4.2 MATERIALS AND METHODS

4.2.1 SMALL SCALE WINE FERMENTATIONS

Shiraz grapes were fermented using different *S. cerevisiae* wine yeast strains during the 2007 harvest season. After destemming and crushing the grape skins, the juice was equally divided into 9 different treatments. All experiments were performed in triplicate. SO_2 was added at a concentration of 20 ppm before fermentation. The grape must was also supplemented with 10 mg/L *ρ*-coumaric acid, 1 mg/L ferulic acid and 1 mg/L caffeic acid to ensure the presence of hydroxycinnamic acid precursors for volatile phenol production. Prior to the onset of alcoholic fermentation, a representative sample of the grape musts of each treatment was taken to determine microbiological status and chemical composition of the musts. The different *S. cerevisiae* and non-*Saccharomyces* commercial yeast strains used in this study are listed in **Table 4.1**. The contribution to volatile phenol production by natural yeasts was monitored by including a spontaneous alcoholic fermentation as control. The rehydration process and inoculation density was used according to the manufacturer's specifications (approximately 10^6 cells/mL). Alcoholic fermentation was conducted at 25°C and the skins were mixed with the juice twice daily. Di-ammonium phosphate was added at a concentration of 0.3 g/L on day three of the fermentation process to ensure sufficient nitrogen levels in the grape juice for successful completion of alcoholic fermentation. Thereafter, the progression of alcoholic fermentation was monitored daily by measuring the decrease in sugar levels with a Brix hydrometer. At the end of alcoholic fermentation the microbial status, chemical composition and the volatile phenol concentrations of the different treatments were determined.

TABLE 4.1
Wine microorganisms evaluated during this study, the experimental media used to test for volatile phenol production, gene fragments tested for by PCR and commercial source of the strains.

Microorganism	Volatile phenol production		PCR screening		Source
	Wine	Synthetic media	*PAD1*	*padA*	
***Saccharomyces* /Non-*Saccharomyces* yeasts**					
Anchor VIN13	√	√	√		Anchor Yeast, South Africa
Anchor WE372	√	√	√		Anchor Yeast, South Africa
Anchor WE14	√	√	√		Anchor, Yeast South Africa
Anchor VIN2000		√	√		Anchor, Yeast South Africa
Anchor N96	√	√	√		Anchor Yeast, South Africa
Lalvin D80	√	√	√		Lallemand, South Africa
Lalvin BM45	√				Lallemand, South Africa
Lalvin D254		√	√		Lallemand, South Africa
Excellence SP	√	√	√		Lamothe Abiet, France
Excellence XR	√	√	√		Lamothe Abiet, France
Melody		√	√		Ch Hansen, Denmark
Symphony		√			Ch Hansen, Denmark
Rhythm		√	√		Ch Hansen, Denmark
Lactic acid bacteria					
L. plantarum 69				√	IWBT, Stellenbosch
L. plantarum 69.1		√		√	IWBT, Stellenbosch
L. plantarum 50		√		√	IWBT, Stellenbosch
L. plantarum 55		√		√	IWBT, Stellenbosch
L. plantarum 73.1		√		√	IWBT, Stellenbosch
L. plantarum 73.2		√		√	IWBT, Stellenbosch
L. brevis 116		√		√	IWBT, Stellenbosch
L. plantarum 43.1				√	IWBT, Stellenbosch
L. plantarum 43				√	IWBT, Stellenbosch
L. plantarum 6.1				√	IWBT, Stellenbosch
L. plantarum 82.2		√		√	IWBT, Stellenbosch
L. plantarum 91		√		√	IWBT, Stellenbosch
L. plantarum 92				√	IWBT, Stellenbosch
L. plantarum 78		√		√	IWBT, Stellenbosch
L. plantarum 75		√		√	IWBT, Stellenbosch
L. plantarum 14.1		√		√	IWBT, Stellenbosch
L. plantarum 14		√		√	IWBT, Stellenbosch
L. pentosus 79.2				√	IWBT, Stellenbosch
Leuconostoc mesenteroides 3.2				√	IWBT, Stellenbosch
O. oeni 152.2				√	IWBT, Stellenbosch
L. brevis 81.1				√	IWBT, Stellenbosch
L. hilgardii 87.1				√	IWBT, Stellenbosch
P. acidilactici 118.2				√	IWBT, Stellenbosch

4.2.2 LABORATORY SCALE FERMENTATIONS

The commercial yeast strains tested for volatile phenols production in synthetic grape juice media are listed in **Table 4.1**. Three of the yeasts used are a combination of *Saccharomyces* and non-*Saccharomyces* yeasts. They include Melody (*S. cerevisiae*; *Kluyveromyces thermotolerans*; *Torulaspora delbrueckii*), Symphony (*S. cerevisiae*; *K. thermotolerans*) and Rhythm (*S. cerevisiae*; *K. thermotolerans*).The synthetic grape juice media (MS300) used during this experiment was prepared according to the protocol of Bely *et al*. (1990) with the supplementation of 10 mg/L *ρ*-coumaric acid, 1 mg/L ferulic acid and 1 mg/L caffeic acid to ensure the presence of hydroxycinnamic acid precursors. One hundred millilitres of synthetic grape juice was transferred into 250 mL Erlenmeyer flasks under sterile conditions and inoculated with 10^6 cells/mL of rehydrated yeast starter culture. Alcoholic fermentation was conducted at room temperature (approximately 25°C). The progression of alcoholic fermentation was monitored by measuring the decrease in mass of each fermentation container and samples were drawn for volatile phenol analysis after day 5 and day 14.

Lactic acid bacterial strains were evaluated for volatile phenols in synthetic wine media containing nutrients and other bacterial requirements (see **Table 4.2**). The media was supplemented with 10 mg/L *ρ*-coumaric acid, 1 mg/L ferulic acid and 1 mg/L caffeic acid to ensure the presence of hydroxycinnamic acid precursors. One hundred millilitres of synthetic wine was transferred into 250 mL sterile Erlenmeyer flasks under sterile conditions. The different LAB strains were pre-cultured in MRS (De Mann Rogosa Sharp) broth (Biolab, Merck, South Africa) and inoculated to the synthetic wine media at approximately 10^6 cells/ml. Incubation occurred at 25°C under static, micro-aerophilic conditions. Volatile phenol levels analysis was performed after 14 days.

TABLE 4.2
Composition of the synthetic wine media[a] (Ugliano *et al*., 2003)

Compounds	Concentrations[b]
Ethanol	12.5 (%v/v)
Tartaric acid	5.0 g/L
L-malic acid	3.5 g/L
Acetic acid	0.6 g/L
D-glucose	2.0 g/L
D-fructose	2.0 g/L
NaCl	0.2 g/L
$(NH_4)_2SO_4$	1.0 g/L
K_2HPO_4	2.0 g/L
$MgSO_4 \cdot 7H_2O$	0.2 g/L
$MnSO_4 \cdot H_2O$	0.056 g/L
Yeast extract	2.0 g/L

[a]pH was adjusted to 3.4 with KOH pellets; medium was sterilised by filtration with 0.22 µm filters.

4.2.3 MOLECULAR DETECTION OF GENES

The yeast strains were screened for the *PAD1* gene by means of colony PCR. Gene specific primers (**Table 4.3**) used for PCR amplification was designed from nucleotide sequences coding for the phenyl acrylic acid decarboxylase gene. PCR was performed in a 50 µL reaction mixture containing a yeast colony (cultured on YPD media), 0.3 µL of each primer, 4 µL $dNTP_s$, 5 µL *Taq* PCR buffer and 0.5 µL *Ex Taq* DNA Polymerase (TaKaRa Biomedicals). The PCR reaction was performed using a PCR Express Thermal Cycler from Hybaid under the conditions indicated in **Table 4.4**.

The LAB were screened by PCR for the *padA* gene as follows: genomic DNA was extracted from the LAB using the method described by Lewington *et al.* (1987). The quantification of DNA was performed spectrophotometrically using a NanoDrop® ND-1000 (NanoDrop Technologies, Inc., Wilmington, USA). The primers (**Table 4.5**) used for PCR amplification were designed from nucleotide sequences coding for the *padA* gene of lactic acid bacteria. Nucleotide sequences used for primer design were obtained from the Integrated Microbial Genomes database. All primers used in this study were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa). PCR was performed in a 50 µL reaction mixture containing 10 ng template DNA, 2 µL of each primer, 5 µL $dNTP_s$, 5 µL *Taq* PCR buffer and 0.25 µL *Ex Taq* DNA Polymerase (TaKaRa Biomedicals). The PCR mixture was cycled using T3 Thermocycler (Biometra) under the conditions indicated in **Table 4.6**.

TABLE 4.3
The primer sequences used for PCR amplification for the *PAD1* gene of wine yeast

Primer name	Primer sequence (5`- 3`)	Reference
Pad-1 (Forward)	GAATTCATGCTCCTATTTCCA	Smit *et al*., 2003
Pad-1 (Reverse)	GATCCTCGAGTTCAATTAGAATGATAT	Smit *et al*., 2003

TABLE 4.4
Thermal cycling conditions used for the PCR detection of *PAD1* gene of wine yeast strains

Primer pair	T_{Di} (°C), time	Main cycling conditions				T_{Ef} (°C), time
		Number of cycles	T_D (°C), time	T_A (°C), time	T_E (°C), time	
Pad1(for)/ Pad1(ref)	94°C,2 min	34	94°C, 30 sec	53°C, 40 sec	72°C, 1 min	72°C,5 min

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

TABLE 4.5
The primer sequences used for PCR amplification for the *padA* gene of lactic acid bacteria

Primer name	Primer sequence (5`- 3`)	Reference
LABPAD1(Forward)	AARAAYGAYCAYACYRTTGATTACC	Oelofse, 2008
LABPAD2(Reverse)	TTCTTCWACCCAYTTHGGGAAAGAA	Oelofse, 2008

TABLE 4.6
Thermal cycling conditions used for the PCR detection of the *padA* gene of lactic acid bacteria

Primer pair	T_{Di} (°C), time	Main cycling conditions				T_{Ef} (°C), time
		Number of cycles	T_D (°C), time	T_A (°C), time	T_E (°C), time	
LABPAD1/ LABPAD2	94°C,2 min	35	94°C,40 s	50°C,1 min s	72°C,30 s	72°C,5 min

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

4.2.4 VOLATILE PHENOL ANALYSIS

All the volatile phenols analysis in this study was performed by Nanosep Laboratories. Briefly, the method used to determine the volatile phenol concentration of wines utilised gas chromatography mass spectroscopy (GC-MS). The analysis included the determination of 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol. A liquid-liquid extraction of 50 mL wine was done with diethyl ether/hexane. An internal standard was included in the analysis. The organic phase was kept and dried over sodium sulphate and then gently concentrated by a low nitrogen flow. Chromatographic analyses were made on a gas chromatograph 3900 equipped with a Saturn 2100T (VARIAN) mass selective detector in a SCAN mode, on a CP WAX 52CB column (30 m x 0.25 mm ID x 0.25 µm film thickness).

4.3 RESULTS AND DISCUSSION

The chemical composition of the Shiraz grape must used for the production of volatile phenols by the commercial wine yeast strains is indicated in **Table 4.7**.

TABLE 4.7
Chemical composition of the Shiraz grape juice used for the small-scale wine fermentation in the 2007 harvest season.

Grape Variety	Region	Harvest season	Sugar conc. (g/L)	Total acidity (g/L)	Tartaric acid (g/L)	pH	Malic acid (g/L)
Shiraz	Franschhoek	2007	254	6.3	5.4	4.04	0.9

4.3.1 VOLATILE PHENOL PRODUCTION BY *S. CEREVISIAE* STRAINS UNDER WINEMAKING CONDITIONS

The alcoholic fermentation rate was monitored through sugar reduction and no differences were observed in the fermentation rate as all the strains finished in 10 days. All the commercial yeast strains evaluated were able to produce 4-vinylphenol and 4-vinylguaiacol in grape juice. From **Figures 4.1** & **4.2** it is clear that the different commercial yeast strains vary in their ability to produce volatile phenols. *S. cerevisiae* yeast strains BM45, D80, VIN13 and N96 produced low levels of 4-VP (75 - 100 µg/L)

and 4-VG (50 - 150 µg/L) respectively. High producers of 4-VP (200 - 325 µg/L) and 4-VG (175 - 350 µg/L) include *S. cerevisiae* SP, WE14, WE372 and XR. Although high standard deviations were evident there is an indication that in the treatments certain yeast strains produced higher concentrations of 4-VP and 4-VG. Chatonnet *et al*. (1993) studied the synthesis of volatile phenols by *S. cerevisiae* during wine production and found that this species was able to transform the hydroxycinnamic acids, *ρ*-coumaric acid and ferulic acid, to 4-VP and 4-VG respectively. They found that high levels of vinyl-derivatives only occur during the fermentation of white wines while red wines typically contain low levels of vinyl-derivatives. In our study it was clear that the total amount of vinyl-derivatives produced by the different strains during red wine fermentation is much lower than found by Chatonnet *et al*. (1993) during the fermentation of white wine. The same authors explained the reason for lower amounts found in red wines is due to the inhibition of the PAD activity of *S. cerevisiae* by polyphenolic compounds in red grapes. Our results are in accordance with Chatonnet *et al*. (1993) who found that alcoholic fermentation of grape must with different strains of *S. cerevisiae* lead to wines with varying levels of 4-VP and 4-VG. *S. cerevisiae* strains therefore vary in their ability to decarboxylate free hydroxycinnamic acids. Van Wyk and Rogers (2000) also investigated the ability of different *S. cerevisiae* yeast strains to decarboxylate ferulic acid to 4-VG in Kerner wines (a white grape varietal). In this study the commercial yeast strains was divided into high (levels between 800 and 954 µg/L) and low (levels between 35 and 600 µg/L) producers of 4-VG. Most of the yeast strains they studied were able to produce much higher levels of 4-VG than found during our study. This confirmed that much higher levels can be produced during the fermentation of white grape juice in comparison to red grape juice. During the evaluation of the different *S. cerevisiae* strains Van Wyk and Rogers (2000) classified the yeast strain WE14 as a low and the yeast strain WE372 as a high producer of 4-VG. Contrary to their results, we found that *S. cerevisiae* WE14 produced higher concentrations of 4-VG in certain instances than *S. cerevisiae* WE372, under our conditions tested. The quality of wine is depreciated if the total amount on 4-VP and 4-VG exceeds 725 µg/L (Chatonnet *et al*., 1993).

The production of high levels of 4-VP and 4-VG by wine yeast strains during alcoholic fermentation could result in higher levels of 4-EP and 4-EG produced by wine microorganisms such as *Brettanomyces* spp. It is therefore suggested that low 4-VP and 4-VG producing commercial *S. cerevisiae* yeast strains should be selected for the fermentation of grape varieties with high levels of hydroxycinnamic acid precursors. This study should be extended to evaluate the production of volatile phenols by other wine yeast strains during the fermentation of different grape varieties; and the possible effect of polyphenolic compounds in red grape juice should also be investigated. In addition, the levels of hydroxycinnamic acid precursors in the South African grape varieties should be determined, as this can determine the degree of risk of volatile phenol production with certain varieties.

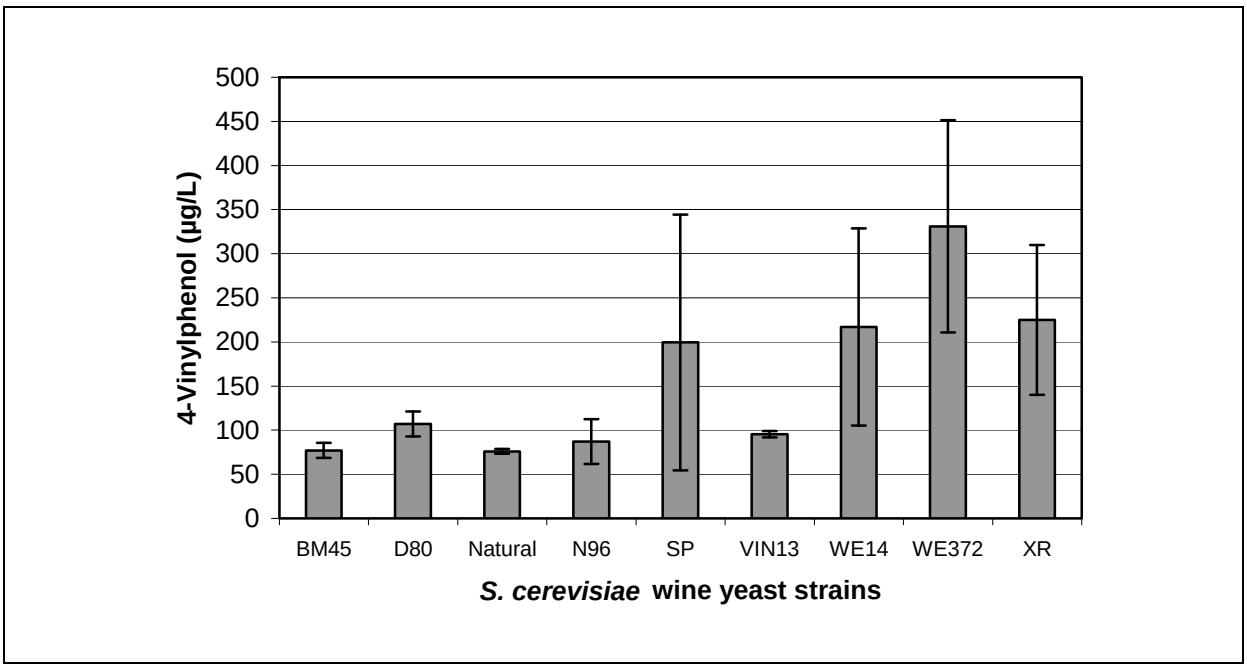

FIGURE 4.1
The production of 4-vinylphenol by different *S. cerevisiae* wine yeast strains during alcoholic fermentation of 2007 Shiraz grapes under normal winemaking conditions. "Natural" denotes the spontaneous alcoholic fermentation performed by native yeast strains (control treatments). Error bars indicate the standard deviation of treatments repeated in triplicate.

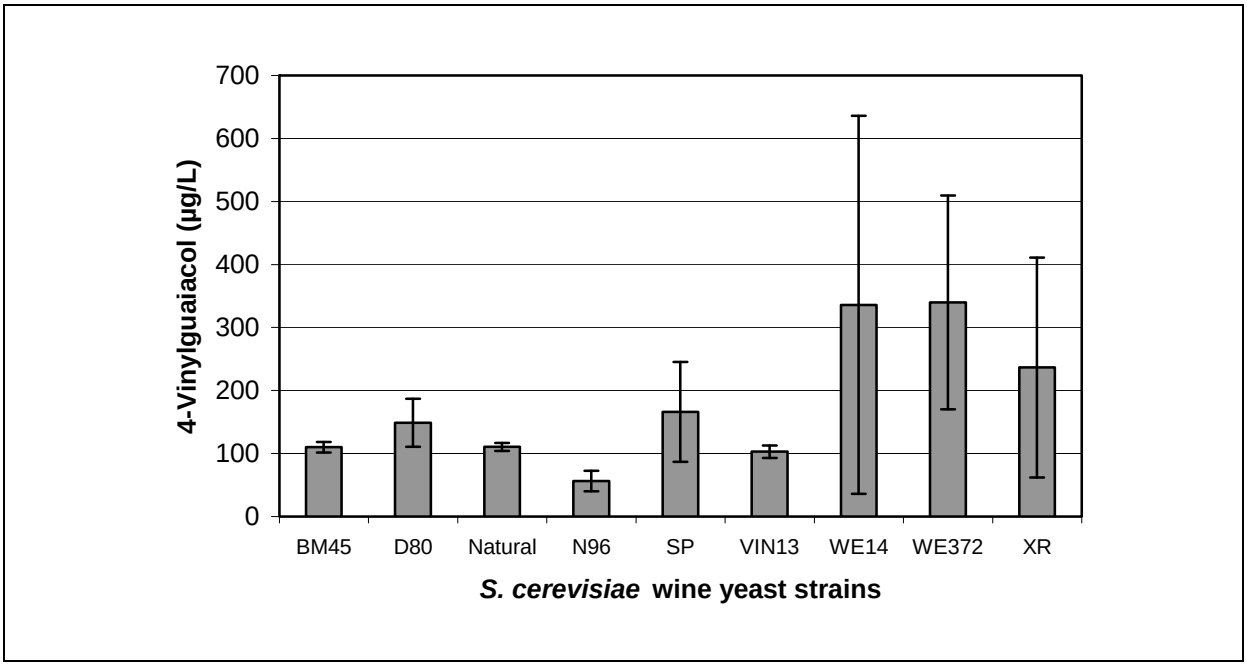

FIGURE 4.2
The production of 4-vinylguaiacol by different *S. cerevisiae* wine yeast strains during alcoholic fermentation of 2007 Shiraz grapes under normal winemaking conditions. "Natural" denotes the spontaneous alcoholic fermentation performed by native yeast strains (control treatments). Error bars indicate the standard deviation of treatments repeated in triplicate.

4.3.2 VOLATILE PHENOL PRODUCTION BY DIFFERENT *S. CEREVISIAE* STRAINS UNDER LABORATORY CONDITIONS

The production of volatile phenols by commercial wine yeast strains under controlled winemaking conditions in synthetic grape juice media (**Figures 4.3** to **4.6**) was much

higher compared to the production under winemaking conditions in Shiraz juice. Considering that wine aroma is depreciated at concentrations higher than 725 µg/L of 4-VP plus 4-VG (Chatonnet *et al*., 1993), *S. cerevisiae* strains WE372, D254, D80, SP, XR, VIN2000 and N96 (with concentrations of 4-VP varying from 800 to 5500 µg/L) can be classified as potential high producers of 4-VP after 5 days of fermentation in synthetic grape juice in this study (**Figure 4.3**). Very high levels of 4-VG were produced by *S. cerevisiae* WE372, D254, D80, SP and N96 yeast strains (concentrations between 600 to 3700 µg/L) after 5 days (**Figure 4.4**). Contrary to the results found with alcoholic fermentation of Shiraz juice the WE14 yeast strain produced low levels of 4-VP and 4-VG in synthetic grape juice compared to the other yeast strains tested. This suggests that a specific yeast strain could vary in their ability to produce volatile phenols during alcoholic fermentation, due to the inhibitory effect of certain compounds present in the red grape must (e.g. high polyphenolic compounds of certain grape varieties).

It is also important to note that even though the Shiraz grape juice probably contained higher levels of *ρ*-coumaric acid and ferulic acid precursors (if we take the natural levels of the grapes into account with the additional added), higher levels of 4-VP and 4-VG was produced in the synthetic media. It has been stated that although red wines have higher levels of phenolic acid precursors they contain lower levels of vinylphenols in comparison to white wines after fermentation (Chatonnet *et al*., 1993). Previously the transformation of vinylphenols to ethylphenols by *Lactobacillus* during malolactic fermentation was considered to be responsible for the lower amounts of vinyl-derivatives found in red wines (Chatonnet *et al*., 1993). Later results showed that the phenyl acrylic acid decarboxylase activity of *S. cerevisiae* was almost totally inhibited by the high levels of polyphenolic compounds found in red wines and therefore lower levels of vinyl-derivatives was produced (Chatonnet *et al*., 1992; Chatonnet *et al*., 1997). Also, in red wine, 4-VP is normally bound to anthocyanins to form stable vinylphenolic anthocyanin colour pigments (Morata *et al*., 2007). These reasons could be an explanation for the higher levels of 4-VP and 4-VG found in the synthetic grape juice media in comparison to red grape juice. Considering that no ethyl derivatives were detected during this study the possibility of the conversion of the vinyl derivatives to ethyl derivatives is excluded.

The ability of the different commercial yeast strains to produce volatile phenols were evaluated after a 14-day period to establish during which stage of fermentation the highest conversion of *ρ*-coumaric acid and ferulic acid to 4-VP and 4-VG occurs. The results show that the stage at which most of the volatile phenols are produced during alcoholic fermentation varies between the different yeast strains (**Figures 4.3 to 4.6**). A possible explanation for this could be that the metabolic activities vary between the different commercial yeast strains and therefore the stages at which the *PAD1* gene is expressed will also be different. The phenyl acrylic acid decarboxylase enzyme is therefore produced at different stages during alcoholic fermentation between the different yeast strains. Another explanation is that the specific enzyme activity can vary between the strains tested.

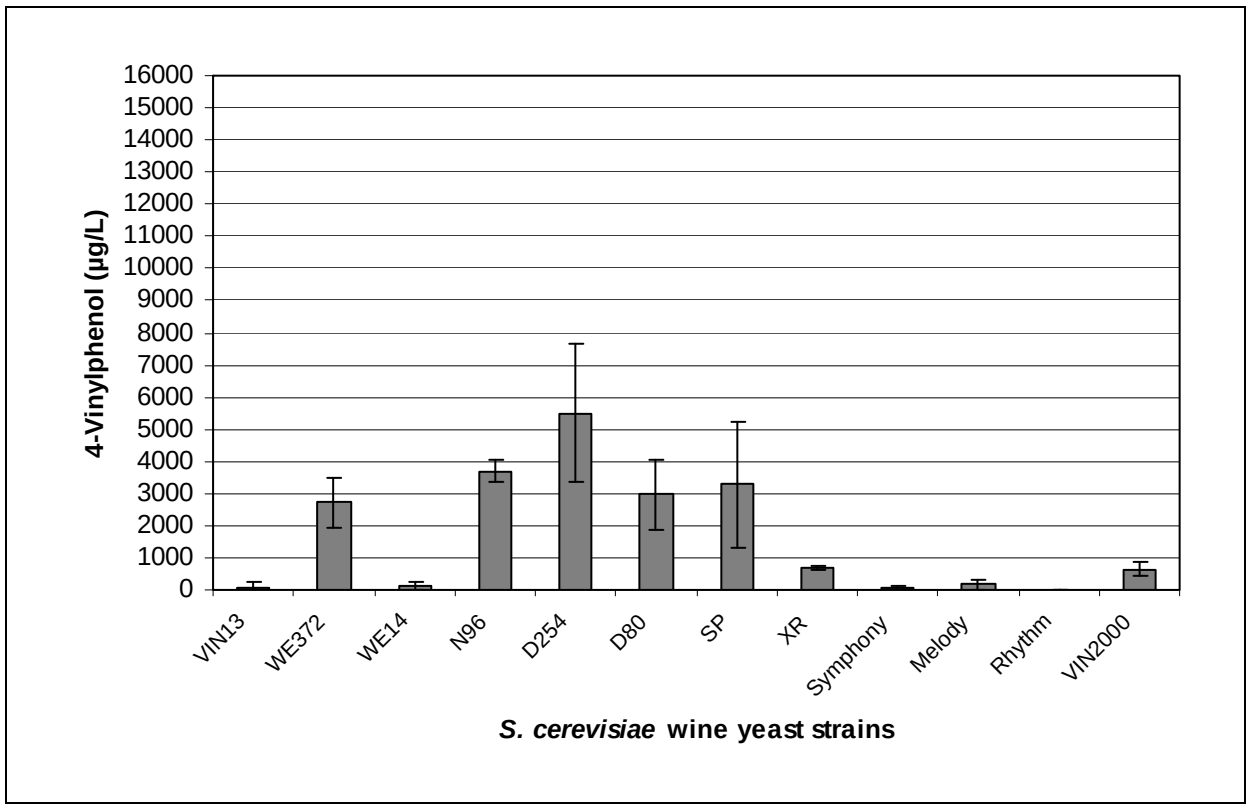

FIGURE 4.3
The production of 4-vinylphenol by different *S. cerevisiae* wine yeast strains during alcoholic fermentation in synthetic grape must under controlled laboratory conditions after five days. Error bars indicate the standard deviation of treatments repeated in triplicate.

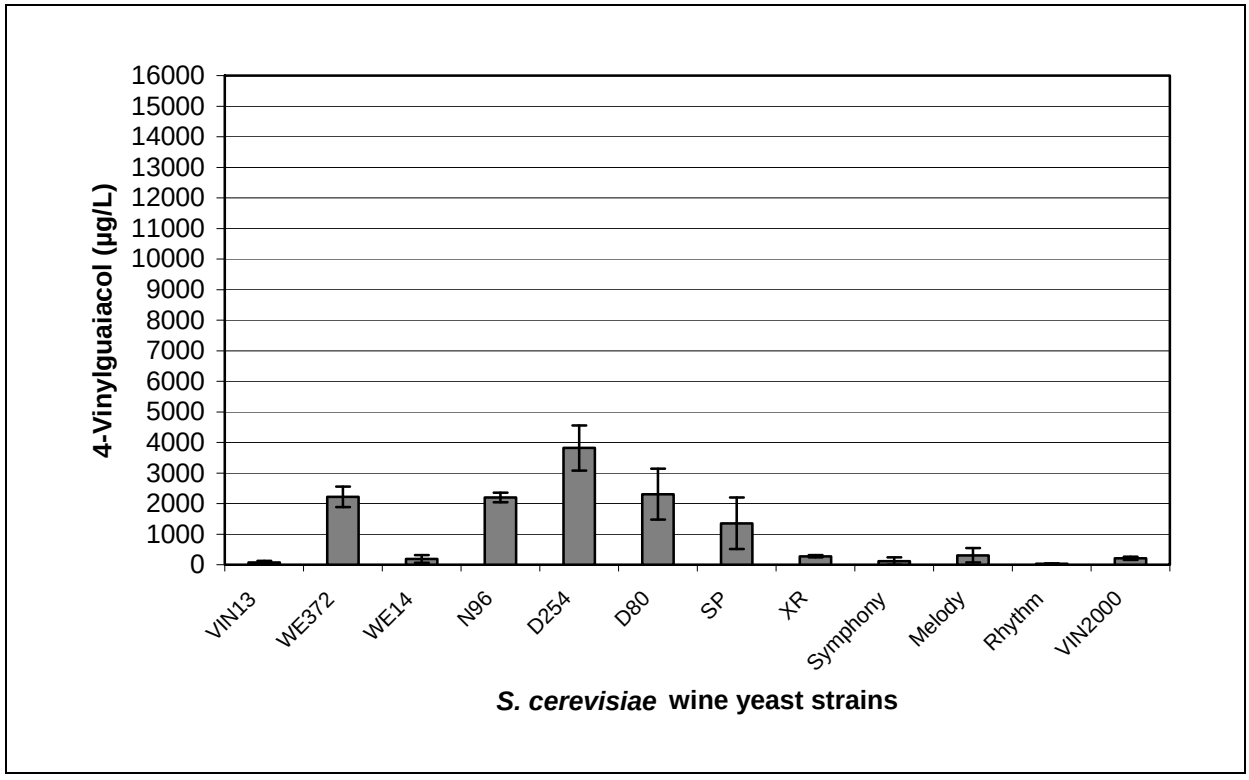

FIGURE 4.4
The production of 4-vinylguaiacol by different *S. cerevisiae* wine yeast strains during alcoholic fermentation in synthetic grape must under controlled laboratory conditions after five days. Error bars indicate the standard deviation of treatments repeated in triplicate.

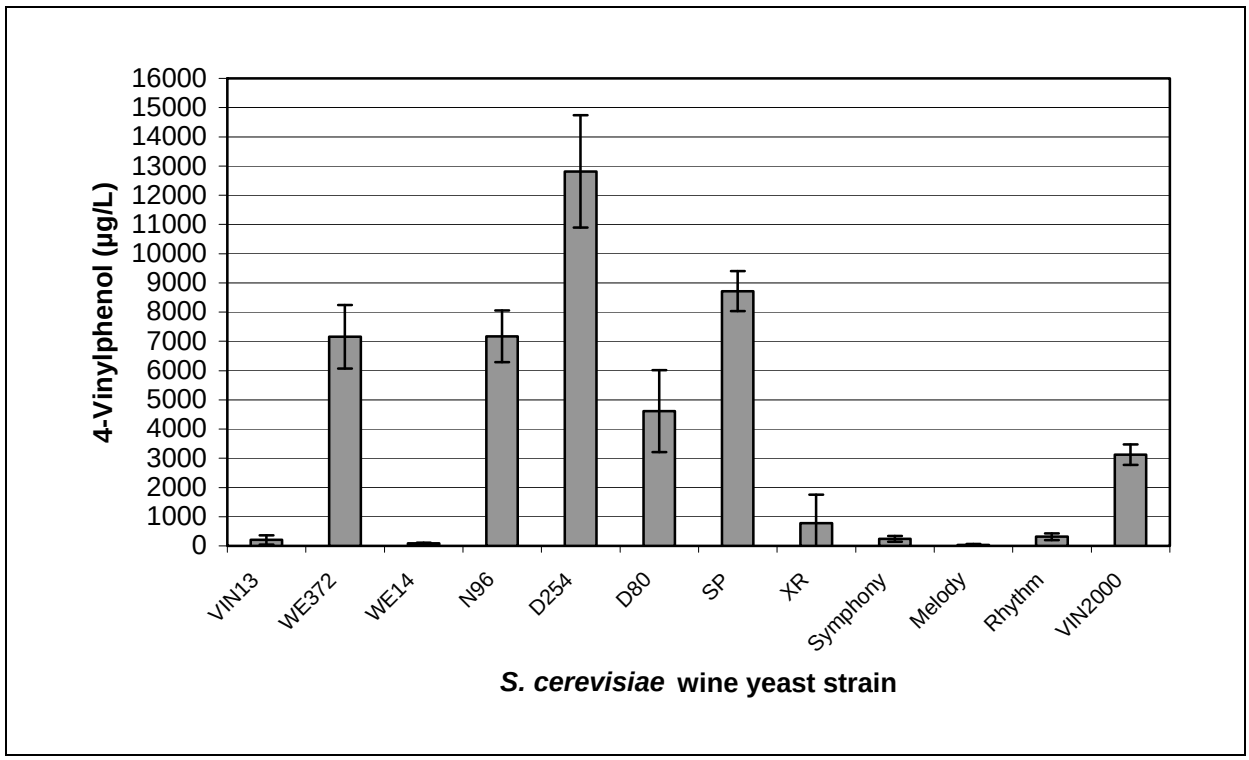

FIGURE 4.5
The production of 4-vinylphenol by different *S. cerevisiae* wine yeast strains during alcoholic fermentation in synthetic grape must under controlled laboratory conditions after fourteen days. Error bars indicate the standard deviation of treatments repeated in triplicate.

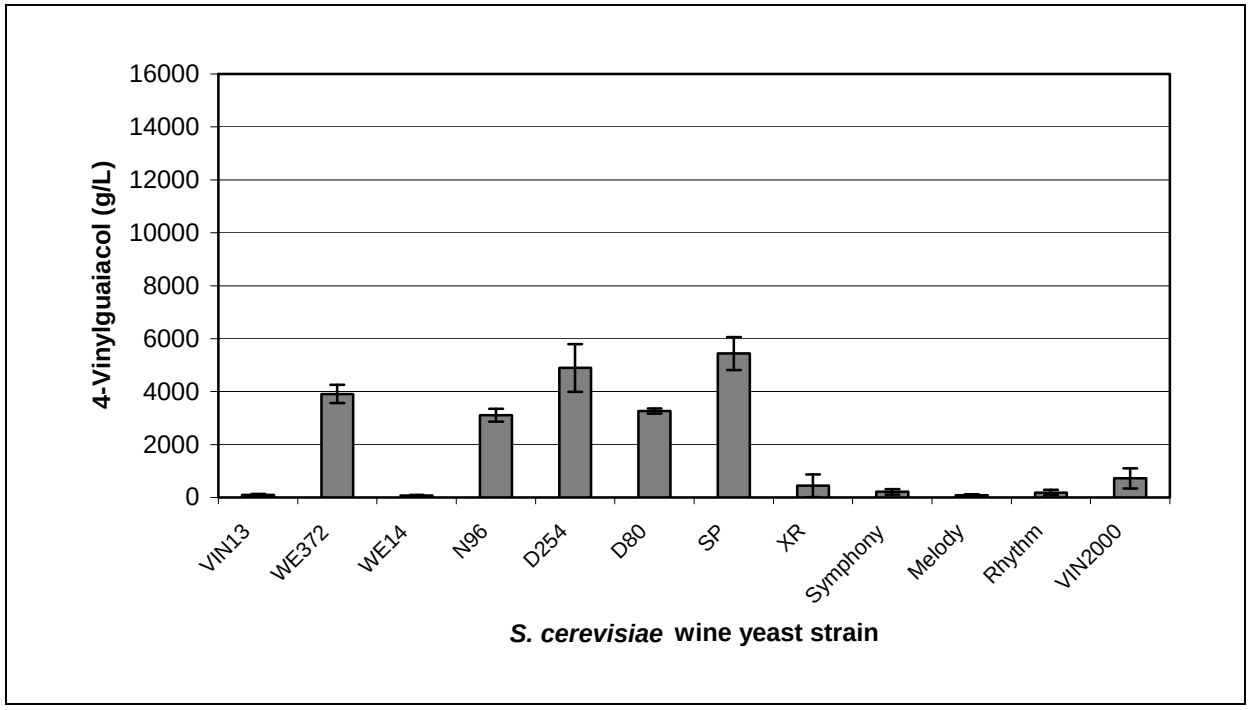

FIGURE 4.6
The production of 4-vinylguaiacol by different *S. cerevisiae* wine yeast strains during alcoholic fermentation in synthetic grape must under controlled laboratory conditions after fourteen days. Error bars indicate the standard deviation of treatments repeated in triplicate.

4.3.3 MOLECULAR DETECTION OF THE PHENYL ACRYLIC ACID DECARBOXYLASE GENE IN *S. CEREVISIAE* WINE YEAST STRAINS

Figure 4.7 shows that the *PAD1* gene could be detected in all the commercial yeast strains evaluated. However, from the results in **Figures 4.3 to 4.6** it is evident that the production of 4-VP and 4-VG varies between the different yeasts. This indicates that the expression of the *PAD1* gene or the substrate specificity of the enzyme varies between the different yeast strains.

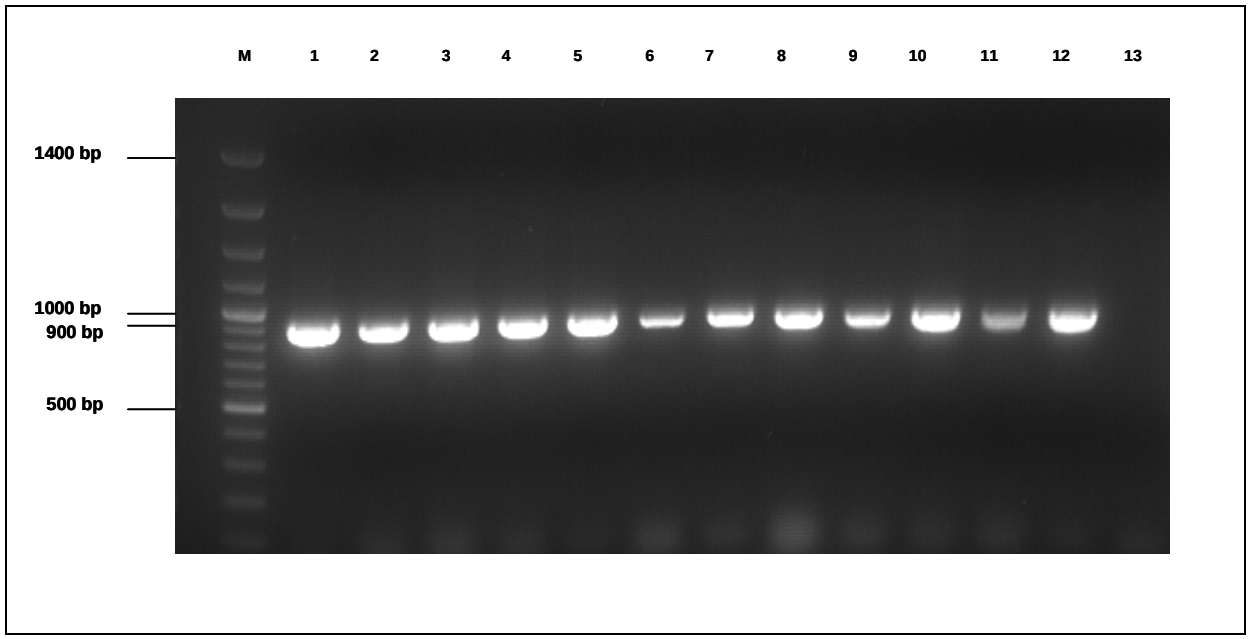

FIGURE 4.7
Molecular detection of the *PAD1* gene in *S. cerevisiae* wine yeast strains by PCR reaction. Lane M: molecular weight marker [GeneRuler^{TM} 100bp DNA Ladder Plus (Fermentas)]. The commercial *S. cerevisiae* wine strains (by lane number) tested: 1, Positive control (VIN13 genomic DNA); 2, VIN13; 3, VIN2000; 4, WE372; 5, WE14; 6, N96; 7, SP; 8, XR; 9, D80; 10, D254; 11, Melody 12, Rhythm; 13, negative control.

4.3.4 MOLECULAR DETECTION OF THE PHENOLIC ACID DECARBOXYLASE GENE IN LACTIC ACID BACTERIA

Of the 23 LAB strains, 12 (52%) tested positive for the presence of the *padA* gene. These are *L. plantarum* 69.1, *L. plantarum* 50, *L. plantarum* 55, *L. plantarum* 73.1, *L. plantarum* 73.2, *L. brevis* 116, *L. plantarum* 82.2, *L. plantarum* 91, *L. plantarum* 78, *L. plantarum* 75, *L. plantarum* 14.1 and *L. plantarum* 14 (Figure 4.8). This indicated that these strains have the possible ability to produce 4-VP and 4-VG from *p*-coumaric acid and ferulic acid, as precursors respectively. However, it is possible that the enzymes might not be active under winemaking conditions.

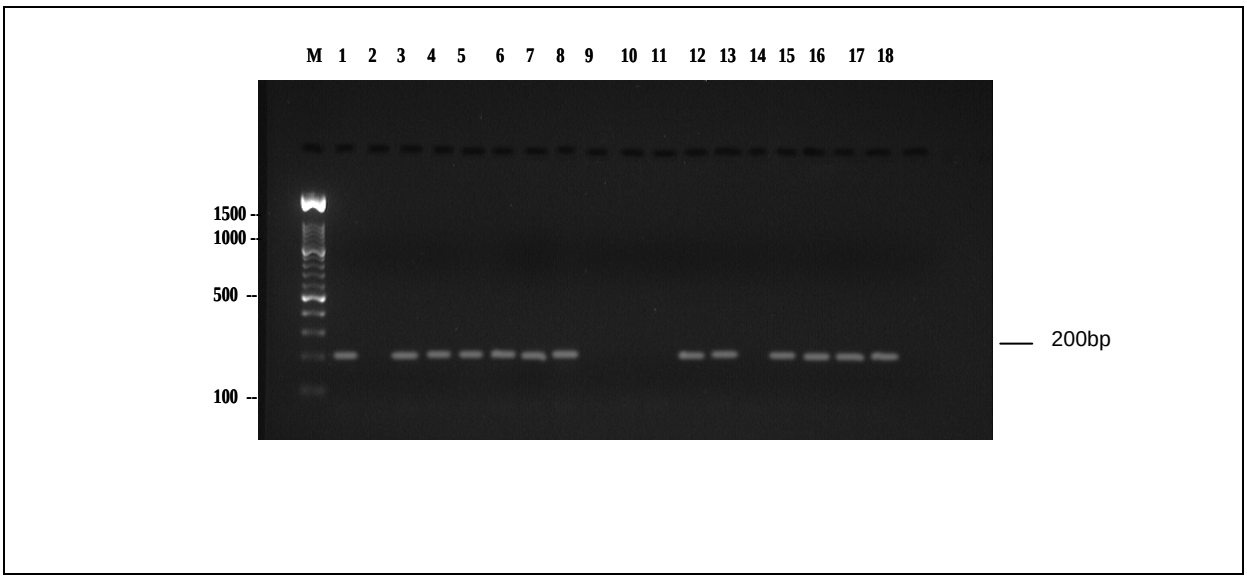

FIGURE 4.8
Molecular detection of the *padA* gene in lactic acid bacteria by PCR reaction. Lane M: molecular weight marker 100 bp-ladder. The lactic acid bacteria isolates (by lane number) tested: 1, Positive control (*E. coli* pjPDC1 with the *padA* locus); 2, *L. plantarum* 69; 3, *L. plantarum* 69.1; 4 *L. plantarum* 50; 5, *L. plantarum* 55; 6, *L. plantarum* 73.1; 7, *L. plantarum* 73.2; 8, *L. brevis* 116; 9, *L. plantarum* 43.1; 10, *L. plantarum* 43; 11, *L. plantarum* 6.1; 12, *L. plantarum* 82.2; 13 *L. plantarum* 91; 14, *L. plantarum* 92; 15, *L. plantarum* 78; 16, *L. plantarum* 75; 17, *L. plantarum* 14.1; 18, *L. plantarum* 14.

4.3.5 VOLATILE PHENOL PRODUCTION BY LACTIC ACID BACTERIA UNDER LABORATORY CONDITIONS

The twelve LAB strains that tested positive for the *padA* gene were evaluated for their ability to produce volatile phenols in synthetic wine media. All the strains except *L. plantarum* 82.2 could produce 4-VP during the fourteen-day incubation period (**Figure 4.9**). Considering that 4-VP plus 4-VG levels higher than 725 µg/L leads to the deterioration of aroma quality (Chatonnet *et al*., 1993), only *L. plantarum* 78 produced levels of 4-VP that far exceeded this threshold and could influence wine quality. The *L. plantarum* 73.1 strain also have the possibility to produce high levels of 4-VP. All the other strains produced very low levels of 4-VP. The production of 4-VG amongst lactic acid bacteria seems to be very low. Only *L. plantarum* 73.1 and *L. plantarum* 78 produced detectable amounts of 4-VG in synthetic wine media (**Figure 4.9**). This could be due to the low amount of ferulic acid precursor added to the media (1 mg/L). None of the strains evaluated produced detectable amounts of 4-EP and 4-EG in synthetic wine media (data not shown).

Cavin *et al*. (1993) also evaluated the ability of wine lactic acid bacteria to metabolise phenolic acids. The authors found that *ρ*-coumaric and ferulic acid were decarboxylated by growing cultures of *L. plantarum, L. brevis* and *Pediococcus* spp. Contrary to our results they found that these strains were able to produce 4-EP and 4-EG under laboratory conditions. A possible explanation could be that they used a different growth media and conditions to evaluate the production of 4-EP and 4-EG. Chatonnet *et al*. (1995) also found that some strains of *L. brevis* and *Pediococcus pentosaceus* were capable of decarboxylating *ρ*-coumaric acid to form 4-VP as actively

as *S. cerevisiae* in a model culture medium. They also noticed that the same bacteria had a lower decarboxylation activity on ferulic acid. It was also found that *L*. *plantarum* could produce detectable quantities of 4-EP in a model culture media. In a later study by Chatonnet *et al*. (1997) it was found that the level of volatile phenols produced by *L. plantarum* in wine was significantly lower in comparison to that produced in a culture media. In their study, lower levels of volatile phenols produced by *L. plantarum* in wine was associated with the inhibitory effect of phenolic compounds, such as tannins, on the decarboxylation activity of this bacterium (Chatonnet *et al*., 1997). In a more recent study by Couto *et al*. (2006) it was found that out of thirty-five strains of LAB screened only thirteen strains were able to produce 4-VP from *ρ*-coumaric acid in culture media. They also found that only *L. brevis*, *L*. *collinoides* and *L*. *plantarum* were able to produce 4-EP.

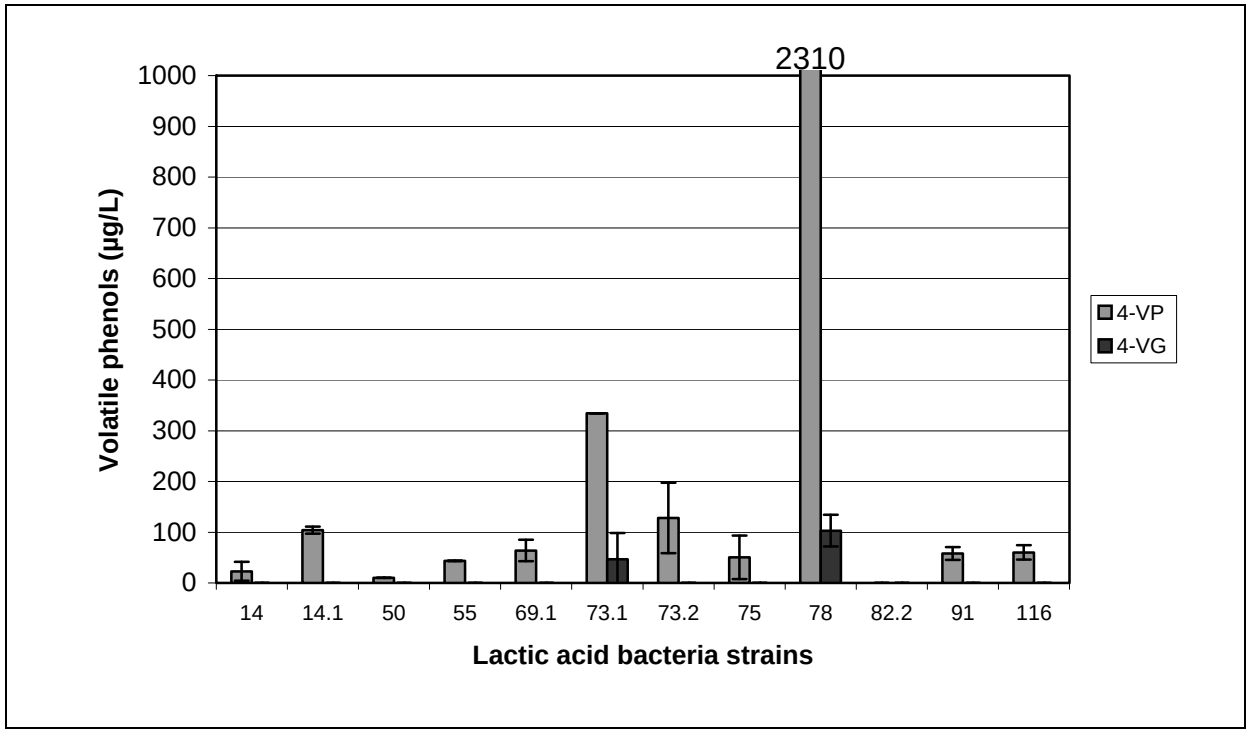

FIGURE 4.9
The production of vinyl-derivatives by lactic acid bacteria in synthetic wine media supplemented with *ρ*-coumaric acid and ferulic acid after fourteen days. Error bars indicate standard deviations of treatments repeated in duplicate.

4.4 CONCLUSIONS

This study showed that commercial yeast strains differ in their ability to produce 4-VP and 4-VG during alcoholic fermentation of red grape must and in synthetic grape must. It is therefore important for winemakers to know that that certain commercial yeast strains could lead to an increased phenolic taint in wines and the use of these yeasts should be avoided especially if contamination by *Brettanomyces* yeasts is a possibility. *Brettanomyces* yeast can convert the vinyl derivatives to the ethyl derivatives and the presence of these compounds causes undesirable aroma characteristics in wine. Overall much higher levels of volatile phenols were formed during the fermentation of

synthetic grape must when compared to natural grape must. This suggests the possible inhibition of the phenyl acrylic acid decarboxylase activity of *S. cerevisiae* yeast by polyphenolic compounds in red grape must. It is therefore a possibility that this activity could vary during the fermentation of different grape varietals (with different polyphenolic compositions) and it is therefore important to note that certain grape varietals might be more prone to the formation of volatile phenols.

Considering that only the *L. plantarum* strains could produce significant levels of 4-VP it is a possibility that spontaneous MLF (dominated by *O. oeni, Lactobacillus* spp. and *Pediococcus* spp.) can lead to higher levels of 4-VP compared to inoculated MLF (dominated by *O. oeni*). The ability of commercial LAB preparations to produce volatile phenols should in future be investigated in detail so that winemakers know which preparation or MLF treatments to perform to minimise the levels of volatile phenols in wines.

4.5 ACKNOWLEDGEMENTS

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Chapter 5

GENERAL DISCUSSION AND CONCLUSIONS

5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 CONCLUDING REMARKS AND FUTURE WORK

Presently there is limited information available on the effect of *Brettanomyces* yeast growth and volatile phenol production in commercial South African wines. To our knowledge, no research has been conducted in South Africa, on the production of volatile phenols during different winemaking practices. This type of information is currently invaluable to winemakers to assist in judicious decisions towards the control of this spoilage yeast. There is also restricted information available on the production of volatile phenols by other wine microorganisms. The production of volatile phenols in commercial South African wines should be regarded as a great concern, considering that in high concentrations these compounds contribute to phenolic-off flavours. It is extremely important for the South African wine industry to ensure that their wines are competitive on an international level and therefore the growth and volatile phenol production by wine microorganisms should be avoided during the winemaking process.

During this study we established a better understanding of the growth and volatile phenol production of *B. bruxellensis* during different stages and with different wine production techniques. We showed that *B. bruxellensis* could survive and produce volatile phenols above the sensory thresholds during alcoholic fermentation. Previously most of the focus was on the volatile phenol production by *Brettanomyces* spp. during wine aging (Chatonnet *et al*., 1992). It was suggested that *Brettanomyces* yeasts populations is present in minor quantities before this time. The explanation for this occurrence was that faster growing wine microorganisms suppressed the growth of these yeasts during alcoholic and malolactic fermentation (MLF) (Chatonnet *et* al., 1995, 1996; Valentão *et al*., 2007). From our results it is clear that proper control of *Brettanomyces* yeast should occur from as early as receiving the grapes to ensure limited growth of these yeasts and volatile phenol production during initial stages of the winemaking process.

Various studies have concentrated on the interaction between *Brettanomyces* yeasts and other wine microorganisms (Dias *et al*., 2003; Renouf *et al*., 2006; Romana *et al*., 2008). We found in our study that alcoholic fermentation performed by two *S. cerevisiae* wine yeast strains used exhibited differences in the *Brettanomyces* spp. populations obtained at the end of alcoholic fermentation. The results indicated that in most cases the *S. cerevisiae* WE14 yeast strains had the ability to produce higher levels of vinyl derivatives in the control treatments. This highlighted the importance of different commercial *S. cerevisiae* yeast strains to produce vinyl derivatives during alcoholic fermentation. A tendency was observed that higher levels of vinyl derivatives could be converted by *Brettanomyces* yeast and consequently leads to higher levels of ethyl derivatives in wine. Therefore caution should be taken when choosing commercial yeast strains to conduct alcoholic fermentation, especially if *Brettanomyces* yeast

contamination is problematic in the winery. The potential production of volatile phenols during alcoholic fermentation by different commercial yeast strains contaminated by *Brettanomyces* yeast deserves further investigation to establish if certain strains pose a greater risk for *Brettanomyces* yeast contamination.

During this study different MLF scenarios were performed to evaluate their influence on the production of volatile phenols in *B. bruxellensis* contaminated wines. There was a tendency in the Cabernet Sauvignon grape varietal to exhibit higher levels of 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) after MLF if *B. bruxellensis* contamination occurred before the onset of alcoholic fermentation. This indicates that if contamination occurs early, the potential spoilage by these yeasts could increase during the winemaking process due to better adaptation in the wine. Also, there was a tendency for wines initially fermented with *S. cerevisiae* WE14 to exhibit higher levels of volatile phenols after MLF. Furthermore, we found that spontaneous MLF can lead to higher levels of volatile phenols in certain cases.

The decarboxylation of phenolic acids by species of LAB, especially *Lactobacillus brevis, Lactobacillus plantarum* and *Pediococcus* spp. has been evaluated (Cavin *et al*., 1993). Considering that these LAB occur naturally and is present during spontaneous MLF they can also possibly contribute to higher levels of volatile phenols (especially 4-VP and 4-VG). Although we initially thought that the presence of lees during MLF could lead to higher levels of volatile phenols produced by *B. bruxellensis*, due to increased availability of nutrients released during yeast autolysis, our results indicated that higher levels was found in some treatments without lees. This could be due to the adsorption of aroma compounds by yeast cell walls (Lubbers *et al*., 1994) and excess levels of volatile phenols were removed by racking the wine from the yeast lees after MLF during our study. Our findings correspond to another study where 4-EP and 4-EG levels could be significantly reduced by the presence of yeast lees (Chassagne *et al*., 2005). More investigations are needed to establish the long-term effect of different MLF practises on the levels of volatile phenols after prolonged aging. Different commercial MLF preparations should also be evaluated for the production of volatile phenols. This type of information is very valuable to winemakers to enable them to make a decision to minimise the levels of volatile phenols produced in wines.

We also found that the Cabernet Sauvignon varietal generally had higher concentrations of ethyl derivatives compared to the Pinotage varietal. This either indicate that the Cabernet Sauvignon varietal have higher concentrations of hydroxycinnamic acid precursors that can be converted to volatile phenols or that during the production of Pinotage wine compounds are produced that inhibit the production of volatile phenols by *B. bruxellensis*. Future investigations should be carried out to determine the levels of volatile phenols produced during the production of different grape varietals. We would then be able to identify if certain varietals pose a higher risk for the production of elevated levels of volatile phenols.

This study also investigated the ability of different commercial wine yeast strains to produce volatile phenols in grape juice and synthetic media after alcoholic fermentation.

All the strains evaluated in grape juice and in synthetic media were able to produce 4-VP and 4-VG during alcoholic fermentation. There was a clear indication that the yeast strains vary in their ability to produce vinyl derivatives. We were also able to successfully establish the presence of the phenyl acrylic acid decarboxylase gene in all the *S. cerevisiae* and non-*Saccharomyces* yeasts tested with PCR screening during this experiment. We also screened 23 strains of LAB by PCR for the presence of the phenolic acid decarboxylase gene. Our results showed that 52% of the strains tested positive for this gene and they were further investigated for their ability to produce volatile phenols in synthetic wine media. Most of the strains were able to produce low levels of 4-VP. *L. plantarum* 78 and *L. plantarum* 73.1 was the only strains that could produce significantly high levels of 4-VP and low levels of 4-VG. This indicates the possible contribution of these strains to elevated levels of vinyl derivatives in wine. This study should however be extended to include more lactic acid bacteria strains and commercial LAB preparations.

To briefly point out the practical value of this work for the wine industry: Firstly *Brettanomyces* yeasts can cause problems during early stages of the winemaking process. Therefore it is important to use healthy or undamaged grapes and also apply winemaking techniques that will lower the chances of developing high levels of volatile phenols in finished wines. Secondly our results conclusively indicated that commercial wine yeast strains could have a major influence on the volatile phenol levels in wines. This suggests that winemakers should choose the correct yeast strains to conduct alcoholic fermentation if lower levels of these compounds are preferred. Thirdly our results also showed that spontaneous MLF could lead to elevated levels of volatile phenols and therefore it is suggested that MLF should rather be induced by commercial preparations of *Oenococcus oeni* bacteria.

Although *Brettanomyces* yeasts are mostly associated with high levels of volatile phenols in wine, the possible contribution by other wine microorganisms should not be excluded. Additional information is needed on the production of volatile phenols by other yeasts and lactic acid bacteria. More studies should be conducted to evaluate the factors and combination of various factors on the production of volatile phenols during winemaking conditions.

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