PCR-BASED DGGE IDENTIFICATION OF BACTERIA AND YEASTS PRESENT IN SOUTH AFRICAN GRAPE MUST AND WINE

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DECLARATION

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

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ABSTRACT

Wine production involves complex interactions between a variety of yeasts and bacteria. Conventional microbiological methods can be used to identify the different microorganisms present in wine, but prove to be time-consuming and certain microbial species may not grow on synthetic isolation media. The aim of this study was to evaluate the microbial population present in two South African red wines, Pinotage and Merlot, as well as five spoilt commercial South African wines by using a non-culturable approach, polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE). The results from the non-culturable approach were compared to conventional platings.

Unique PCR-based DGGE fingerprints were obtained for the *Bacteria* and yeasts present in the South African Pinotage and Merlot wines. Using yeast specific primers the Pinotage wine showed the presence of non-*Saccharomyces* yeasts at the beginning of the alcoholic fermentation, while *Saccharomyces cerevisiae* was present until the completion of the malo-lactic fermentation (MLF). This yeast was also identified during both the alcoholic fermentation and MLF of the Merlot wine using PCR-based DGGE and conventional plating. Using *Bacteria* specific primers, *Lactobacillus plantarum* and *Lactobacillus* sp. was identified in the Pinotage wine using PCR-based DGGE, while *Lactobacillus brevis* were isolated from Merlot wine using conventional platings.

Although the presence of *S. cerevisiae* is expected during wine fermentation, the presence of this microbe in bottled wine could lead to spoilage. Four of the spoilt commercial wine samples (RW1, RW2, RoW1 and WW1) were found to be spoilt by *S. cerevisiae*, while a fifth wine sample (RW3) was found to be spoilt by an *Acetobacter* sp. using PCR-based DGGE.

Members of the family *Enterobacteriaceae* were identified from all the wines using PCR-based DGGE, while *Enterobacter sakazakii* was identified from RW1 using PCR-based DGGE and conventional plating. The members of the family *Enterobacteriaceae* could possibly have contributed to the spoilage of the wine by producing undesirable secondary metabolites.

PCR-based DGGE proved to be an alternative to conventional microbiological methods for the identification of the microbial species in South African red grape must and wine. This method also proved to be useful in the identification of spoilage microbes in spoilt commercial South African wines.

UITTREKSEL

Die produksie van rooi wyn behels komplekse interaksies tussen ‘n verskeidenheid van giste en bakterieë. Konvensionele mikrobiologiese metodes kan gebruik word om die verskillende mikro-organismes wat in rooi wyn teenwoordig is te identifiseer, maar dit blyk tydrowend te wees, terwyl sekere mikro-organismes nie groei op sintetiese media nie. Die doel van hierdie studie was om die mikrobiologiese populasie wat in twee Suid-Afrikaanse rooi wyne, Pinotage en Merlot, en vyf bederfde kommersiële wyne teenwoordig is, te evalueer met die gebruik van ‘n kultuur-onafhanklike benadering, polimerase ketting-reaksie (PKR)-gebaseerde denaturerende gradiënt jel elektroforese (DGJE). Die resultaat van die kultuur-onhafhanklike benadering was vergelyk met konvensionele uitplating tegnieke.

Unieke, ongeëwenaarde PKR-gebaseerde DGGE vingerafdrukke was verkry van die *Bakterieë* en giste aanwesig in die Pinotage en Merlot wyne. Deur gebruik te maak van gis-spesifieke inleiers het die Pinotage wyn die teenwoordigheid van nie-*Saccharomyces* giste getoon, terwyl *Saccharomyces cerevisiae* teenwoordig was tot en met die afhandeling van die appel-melksuur gisting (AMG). Hierdie gis is ook geïsoleer gedurende beide die alkoholiese gisting en AMG van die Merlot wyn deur gebruik te maak van PKR-gebaseerde DGGE en konvensionele uitplating tegnieke. Met *Bakterieë*-spesifieke inleiers, was *Lactobacillus plantarum* en *Lactobacillus* sp. geïdentifiseer in die Pinotage wyn deur gebruik te maak van PKR-gebaseerde DGGE, terwyl *Lactobacillus brevis* geïsoleer is uit Merlot wyn deur gebruik te maak van konvensionele uitplatings.

Alhoewel die teenwoordigheid van *S. cerevisiae* verwag word gedurende wynfermentasie, kan die teenwoordigheid van hierdie mikrobe in gebottelde wyn tot bederwing lei. Vier van die bedorwe kommersiële wynmonsters (RW1, RW2, RoW1 en WW1) was bederf deur *S. cerevisiae*, terwyl ‘n vyfde wynmonster (RW3) bederf was deur ‘n *Acetobacter* sp. deur die gebruik van PKR-gebaseerde DGGE.

Van al die wyne is lede van die *Enterobacteriaceae* familie geïdentifiseer deur gebruik gemaak te maak van PKR-gebaseerde DGGE, terwyl *Enterobacter sakazakii* geïsoleer is van RW1 met konvensionele uitplating. Die lede van die familie

Enterobacteriaceae kon moontlik bygedra het tot die bederwing van die wyn deur ongewenste sekondêre metaboliete te produseer.

PKR-gebaseerde DGGE bewys ‘n alternatief tot die konvensionele mikrobiologiese metodes vir die identifikasie van die mikrobiese spesies in Suid-Afrikaanse rooi druif mos en wyn te wees. Hierdie metode het ook die bruikbaarheid in die identifikasie van mikrobes wat kommersiële Suid-Afrikaanse wyne bederf, bewys.

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Without faith you can do nothing, with faith anything is possible

- Sir William Osler

Dedicated to my husband, Pieter

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Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

The microbiology of wine fermentation was first reported by Louis Pasteur in the 1850s when he observed the conversion of grape juice into wine by the metabolism of yeasts. However, winemaking is a far more complex biochemical process involving interactions between a variety of different yeasts, bacteria and mycelial fungi (Fleet, 1993; Du Toit & Pretorius, 2000). Although the grape variety influences the wine flavour, quality and aroma, it is the quantity and variety of microorganisms that occur throughout the fermentation process that characterise the wine (Fleet, 2003). The microorganisms involved in winemaking are non-*Saccharomyces* yeasts, which include *Hanseniaspora*, *Candida*, *Metschnikowia*, *Hansenula*, *Zygosaccharomyces*, *Brettanomyces*, *Aureobasidium*, *Rhodotorula*, *Pichia*, *Kluyveromyces*, *Cryptococcus*, *Dekkera*, *Schizosaccharomyces*, *Torulaspora* and *Saccharomycodes*, as well *Saccharomyces cerevisiae* (Fleet & Heard, 1993; Fugelsang, 1997; Fleet, 2003; Querol *et al*., 2003; Romano *et al*., 2003). The alcoholic fermentation conducted by the yeasts is followed by a malo-lactic fermentation (MLF), carried out by lactic acid bacteria (LAB), including species of *Lactobacilllus, Pediococcus* and *Oenococcus* (Henick-Kling, 1993; Fugelsang, 1997). Although all these microbes are associated with the winemaking process, some can be considered spoilage organisms when metabolic by-products exceeding legal or sensory limits are produced (Rapp & Versini, 1991; Lambrechts & Pretorius, 2000; Fleet, 2003).

Another group of bacteria associated with wine spoilage are the acetic acid bacteria (AAB) which include *Gluconobacter oxydans, Acetobacter aceti*, *Acetobacter pasteurianus, Gluconacetobacter liquefaciens* and *Gluconacetobacter hansenii* (Du Toit & Lambrechts, 2002). The AAB can raise the level of acetic acid in the wine to unacceptable levels, known as the acetification of wine (Drysdale & Fleet, 1988).

Methods used for the detection and identification of these microbes in must and wine includes conventional microbiological plating methods and molecular approaches. Conventional microbiological plating methods, involving selective cultivation and isolation of microbes are widely used to identify the different yeast and

bacterial species present in wine (Fleet & Heard, 1993; Dias *et al*., 2003; Medawar *et al*., 2003; Pasteris & Strasser de Saad, 2005; Ciani *et al*., 2006; Pérez-Nevado *et al*., 2006; Renouf & Lonvaud-Funel, 2006), as well as to identify spoilage yeasts, LAB and AAB (Ubeda & Briones, 1999). However, these plating methods are time-consuming and not all microbes can be cultured on synthetic growth media (Heard & Fleet, 1986; Kopke *et al*., 2000). Molecular techniques, such as polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) have proven to be reliable and rapid alternatives for conventional microbiological plating (Ercolini, 2004). This technique has emerged as a powerful diagnostic tool for the direct profiling of the microbial diversity present in wine without the need for cultivation (Cocolin *et al*., 2000; Kawai *et al*., 2002; Lopez *et al*., 2003; Prakitchaiwattana *et al*., 2004).

The aim of this study was to identify the microbial population present in South African red grape must and wine, as well as spoilt commercial wines using PCR-based DGGE fingerprinting and DNA sequencing. This technique was also compared to conventional microbiological plating methods.

References

Ciani, M., Beco, L. & Comitini, F. (2006). Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *International Journal of Food Microbiology*, **108**, 239-245.

Cocolin, L., Bisson, L.F. & Mills, D.A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiology Letters*, **189**, 81-87.

Dias, L., Dias, S., Sancho, T., Stender, H., Querol, A., Malfeito-Ferreira, M. & Loureiro, V. (2003). Identification of yeasts isolated from wine-related environments and capable of producing 4-ethylphenol. *Food Microbiology*, **20**, 567–574.

Du Toit, M. & Pretorius, I.S. (2000). Microbial spoilage and preservation of wine: using weapons from nature's own arsenal – a review. *South African Journal of Enology and Viticulture*, **21**, 74-96.

Du Toit, W.J. & Lambrechts, M.G. (2002). The enumeration and identification of acetic acid bacteria from South African red wine fermentations. *International Journal of Food Microbiology*, **74**, 57-64.

Drysdale, G.S. & Fleet, G.H. (1988). Acetic acid bacteria in winemaking: a review. *American Journal of Enology and Viticulture*, **39**, 143-152.

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, **56**, 297-314.

Fleet, G.H. (1993). The microorganisms of winemaking – isolation, enumeration and identification. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 1-26. New York: Taylor & Francis.

Fleet, G.H. & Heard, G.M. (1993). Yeasts-growth during fermentation. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 27-54. New York: Taylor & Francis.

Fleet, G.H. (2003). Yeast interactions and wine flavour. *International Journal of Food Microbiology*, **86**, 11-22.

Fugelsang, K.C. (1997). Wine Microbiology. Pp. 3-47, 48-67, 68-111, 132-137. New York: Chapman & Hall.

Heard, G.M. & Fleet, G.H. (1986). Evaluation of selective media for enumeration of yeasts during wine fermentation. *Journal of Applied Bacteriology*, **60**, 477-481.

Henick-Kling, T. (1993). Malo-lactic Fermentation. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 289-326. New York: Taylor & Francis.

Kawai, M., Matsutera, E., Kanda, H., Yamaguchi, N., Tani, K. & Nasu, M. (2002). 16S Ribosomal DNA-based analysis of bacterial diversity in purified water used in pharmaceutical manufacturing processes by PCR and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, **68**, 699-704.

Kopke, C., Cristovão, A., Prata, A.M., Silva Pereira, C., Figueiredo Marques, J.J. & San Romão, M.V. (2000). Microbiological control of wine. The application of epfiluorescence microscopy method as a rapid technique. *Food Microbiology*, **17**, 257-260.

Lambrechts, M.G. & Pretorius, I.S. (2000). Yeast and its importance to wine aroma – a review. *South African Journal of Enology and Viticulture*, **21**, 97-129.

Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., VanderGheynst, J. & Mills, D.A. (2003). Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, **69**, 6801-6807.

Medawar, W., Strehaiano, P. & Délia, M. (2003). Yeast growth: lag phase modeling in alcoholic media. *Food Microbiology*, **20**, 527–532.

Pasteris, S.E. & Strasser de Saad, A.M. (2005). Aerobic glycerol catabolism by *Pediococcus pentosaceus* isolated from wine. *Food Microbiology*, **22**, 399–407.

Pérez-Nevado, F., Albergaria, H., Hogg, T. & Girio F. (2006). Cellular death of two non-Saccharomyces wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. *International Journal of Food Microbiology*, **108**, 336–345.

Prakitchaiwattana, C.J., Fleet, G.H. & Heard, G.M. (2004). Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Research*, **4**, 865-877.

Querol, A., Fernández-Espinar, M.T., lí del Olmo, M. & Barrio, E. (2003). Adaptive evolution of wine yeast. *International Journal of Food Microbiology*, **86**, 3-10.

Renouf, V. & Lonvaud-Funel, A. (2006). Development of an enrichment medium to detect *Dekkera*/*Brettanomyces bruxellensis*, a spoilage wine yeast, on the surface of grape berries. *Microbiological Research*, **article in press**.

Rapp, A. & Versini, G. (1991). Influence of nitrogen compounds in grapes on aroma compounds in wine. In: *Proceedings of the International symposium on nitrogen in grapes and wines* (edited by RANTZ). Pp. 156-164. Davis, CA: American Society for Enology and Viticulture.

Romano, P., Fiore, C., Paraggio, M., Caruso, M. & Capece, A. (2003). Function of yeast species and strains in wine flavour. *International Journal of Food Microbiology*, **86**, 169-180.

Ubeda, J.F. & Briones, A.I. (1999). Microbiological quality control of filtered and non-filtered wines. *Food Control*, **10**, 41-45.

CHAPTER 2

LITERATURE REVIEW

A. Background

Fermentation is known to be one of the oldest methods of food preservation, while also contributing significantly to the flavour, aroma and texture of the end-product. Fermentation refers to the utilization of the natural sugars in foods by yeasts and lactic acid bacteria (LAB), producing alcohol and carbon dioxide as metabolic by-products. The alcohol in turn creates an environment in which the growth of spoilage organisms is limited. Fermented food products include ripened cheese, pickles, sausages, and fermented beverages, such as wine and beer (Fugelsang, 1997; Jay, 1998).

Louis Pasteur first reported the microbiology of wine fermentation in the 1850s when he observed the conversion of grape must into wine by yeast metabolism. However, wine fermentation is a far more complex biochemical process involving interactions between a variety of different yeasts, bacteria and mycelial fungi (Fleet, 1993; Du Toit & Pretorius, 2000). The quality, aroma and flavour of the wine is greatly influenced by the quantity and variety of microorganisms that occur throughout the fermentation process (Fleet, 2003).

B. Wine fermentation

Two fermentation stages can occur during the production of wine, namely an alcoholic fermentation, followed by a malo-lactic fermentation (MLF) (Boulton *et al*., 1996). Alcoholic fermentation represents the main fermentation step in the production of wine, where the fermentable sugars present in the grapes are converted to ethanol and carbon dioxide (CO_2). The sugars in the grapes are released by crushing the grapes and ridding them of the stalks. This mixture, referred to as the grape must, can undergo spontaneous fermentation by the microbes present on the grapes and those prevalent in

the winery environment (Fleet & Heard, 1993). Yeasts and bacteria native to grapes can yield wine with distinctive sensory attributes described as being “fuller” and “rounder”. However, natural fermentations will yield wines of varying sensory quality, since the type and number of microbes present can vary. The natural fermentation may take longer to reach completion due to lower microbial counts. Therefore, many winemakers choose to inoculate the must with active, dry wine yeast strains of *Saccharomyces cerevisiae* at a concentration of *ca* 1 - 3 x 10^6 cfu.ml^{-1} (Fugelsang, 1997; Nikolaou *et al*., 2005). In red wine processing, both the spontaneous and inoculated alcoholic fermentation processes take place at temperatures between 20° - 30°C. The wine is then pressed from the skins, drawn from the sediment and aged (Fugelsang, 1997).

MLF takes place two to three weeks after the end of the alcoholic fermentation, during which L(-)-malic acid is converted to L(+)-lactic acid and CO_2 (Henick-Kling, 1993). It is a process that de-acidifies the wine and is carried out by LAB, either spontaneously or by inoculation with commercial starter cultures of *Oenococcus oeni* (Henick-Kling, 1993; Lonvaud-Funel, 1995; Lonvaud-Funel, 1999). This process is significant in wines produced from grapes grown in cooler climates, which often have a low pH and a high acid content (Henick-Kling, 1993; Alexandre *et al*., 2004).

MLF is often encouraged as it improves the aroma, flavour and microbial stability of the wine. Microbial stability is accomplished by the depletion of remaining nutrients, the production of lactic acid which acts as an antimicrobial agent and the possible production of bacteriocins by the LAB. The result is wine with restricted growth of fastidious microorganisms (Henick-Kling, 1993; Boulton *et al*., 1996; Alexandre *et al*., 2004).

The flavours associated with MLF are described as “buttery”, “nutty”, “oaky”, and “sweaty”. It augments the fruity character of the wine, reduces the vegetative, green flavour from red wines produced in cooler climates and the taste of malic acid disappears (Henick-Kling, 1993). Through their metabolic activity on anthocyanins, the LAB also modifies the colour of the wine (Lonvaud-Funel, 1995).

Even though MLF is favourable in wines grown in cooler climates, it can under specific conditions be considered as a spoilage process. In warmer regions where less

acidic wines are produced, the pH increasing characteristic of MLF can adversely influence the microbial stability and the quality of the wine, resulting in a wine with too little acidity (Henick-Kling, 1993). The reduced acidity may also lead to a *ca* 30% reduction of the red colour of the wine (Van Vuuren & Dicks, 1993).

C. Microbial populations in wine

The formation of flavour and aroma is very complex in wine. The most flavour compounds are formed during alcoholic fermentation by yeasts present in the must and wine which can originate from three sources, namely the grape surface, winery equipment and the starter inoculum (Fleet & Heard, 1993). The flavour precursors on the grapes and the microbes present during maturation, also have an influence on the organoleptic quality of the wine (Nykänen, 1985). Although the grape variety influences the wine flavour, it is the interactions between the different microbial species and the fermentation conditions that characterise the wine flavour profile and quality (Fleet, 2003). These microbial metabolic by-products include ethanol and CO_2, as well as many other secondary products. All these compounds influence the character of the wine either positively or negatively and it is therefore important to understand the interactions of the different microbes during the winemaking process (Rapp & Versini, 1991; Lambrechts & Pretorius, 2000; Fleet, 2003).

Yeasts

Yeasts are significant in determining the wine quality and influences the wine flavour through altering the existing aromatic precursors in the grape must or by producing new aromatic compounds. These reactions vary with the species and strains of yeasts present in the must and wine (Fleet, 2003; Romano *et al*., 2003). Through their metabolism and interactions, the yeasts produce either desirable or undesirable compounds during fermentation. Desirable compounds include higher alcohols, esters, organic acids and acetaldehydes (Rapp & Versini, 1991). Higher alcohols have a high

molecular weight and are considered to be the largest group of the aroma compounds (Nykänen, 1985). At concentrations below 300 $mg.L^{-1}$ they can impart desirable notes, but above 400 $mg.L^{-1}$ they are considered to have a negative influence on the aroma of the wine (Rapp & Mandery, 1986).

Esters are volatile compounds that give pleasant aromas, such as “fruitiness” and “floweriness” to the wine (Lambrechts & Pretorius, 2000). Ethyl acetate is the major ester compound associated with wine, but it can encourage spoilage when present at levels exceeding 200 $mg.L^{-1}$, especially when coupled with acetic acid at concentrations of approximately 600 $mg.L^{-1}$ (Fugelsang, 1997). Acetic acid contributes to more than 90% of the volatile acidity in wine and is formed early in the fermentation when the sugar concentration is high (Radler, 1993). Although it adds to the volatile acidity of the wine, spoilage occur when acetic acid is present at concentrations of 1.2 – 1.3 $g.L^{-1}$, giving the wine a vinegary taint (Sponholz, 1993).

Acetaldehyde is highly volatile and if it is present in excessive amounts, it produces a “bruised apple” aroma in the wine (Fugelsang, 1997). Yeasts may contribute up to 75 $mg.L^{-1}$ of acetaldehyde during alcoholic fermentation, and as it can also be produced by acetic acid bacteria (AAB), it can be present in aging wines with an aroma threshold of 100 – 120 $mg.L^{-1}$. Spoilage occurs when concentrations above 160 $mg.L^{-1}$ are reached (Fugelsang, 1997).

Compounds such as hydrogen sulfide (H_2S), volatile phenols and high concentrations of diacetyl produced by yeasts are undesirable in wine (Boulton *et al*., 1996). Although diacetyl spoilage mostly occurs during MLF, it can also be produced by yeasts (Fugelsang, 1997). Diacetyl is a flavour compound found in butter and dairy products, but it is also present in wine, brandy, roasted coffee and other fermented food products (Bartowsky & Henschke, 2004). It gives a buttery aroma to the wine when present at levels exceeding 0.4 $mg.L^{-1}$ (Fugelsang, 1997). The off-flavours associated with rotten eggs, rubber, onion, garlic, cabbage and skunks are associated with sulphur compounds such as H_2S (Boulton *et al*., 1996). It has a low sensory threshold level of 10 – 100 $\mu g.L^{-1}$ and its formation is associated with a deficiency of nitrogen in the grape must (Lambrechts & Pretorius, 2000). The most significant volatile phenols in red wine

are the ethyl-derivatives (4-ethylguiacol and 4-ethylphenol) that impart off-odours associated with “horse sweat”, “stables” and “medicinal” smells. These aromas are detectable above concentrations of 100 $\mu g.L^{-1}$ for 4-ethylguiacol and 600 $\mu g.L^{-1}$ for 4-ethylphenol (Chatonnet *et al*., 1992).

Non-Saccharomyces yeasts

Various yeast species are associated with grapes, must and wine. Although *Saccharomyces cerevisiae* dominate the alcoholic fermentation process, it is not commonly isolated from grapes. Healthy grapes contain a selection of different yeasts that play significant roles in the final quality of the wine and these are known as the non-*Saccharomyces* yeasts (Fugelsang, 1997; Fleet, 2003). The non-*Saccharomyces* yeasts include species of the genera *Hanseniaspora*, *Candida*, *Metschnikowia*, *Hansenula*, *Zygosaccharomyces*, *Brettanomyces*, *Aureobasidium*, *Rhodotorula*, *Pichia*, *Kluyveromyces*, *Cryptococcus*, *Dekkera*, *Schizosaccharomyces*, *Torulaspora* and *Saccharomycodes* (Fleet & Heard, 1993; Fugelsang, 1997; Fleet, 2003; Querol *et al*., 2003; Romano *et al*., 2003).

These non-*Saccharomyces* yeasts are present on the grapes prior to harvest and they are also present in the grape must and the early stages of alcoholic fermentation (Fleet & Heard, 1993). Their survival during the fermentation depends on the alcohol concentration (Fleet & Heard, 1993; Lambrechts & Pretorius, 2000). The non-*Saccharomyces* yeasts initiate the alcoholic fermentation process, but die-off within the first two to three days (Fleet & Heard, 1993), since they are not tolerant to ethanol concentrations higher than 5 - 7% (v/v). At higher ethanol concentrations the principle wine yeast *S. cerevisiae* dominates the alcoholic fermentation (Fleet & Heard, 1993). Apart from the alcohol concentration, the metabolic patterns of the non-*Saccharomyces* yeasts are also influenced by the temperature at which fermentation takes place. Low fermentation temperatures between 10° - 15°C can increase the alcohol tolerance of *C. stellata* and *Hanseniaspora* spp., resulting in their presence later on during the fermentation process (Fugelsang, 1997; Fleet, 2003). This can in turn influence the organoleptic quality of the wine, since *Candida* spp. and *Hansenula* spp. can produce

approximately 100 times more ethyl acetate in an anaerobic environment when ethanol is present (Plata *et al*., 2003). Other factors influencing the survival of these yeasts include available nutrients, sulphur dioxide (SO_2) concentration and the initial variety of microbes present on the grapes (Fleet & Heard, 1993; Lambrechts & Pretorius, 2000).

Brettanomyces species represent one of the most important spoilage yeasts associated with wine (Sponholz, 1993). Species of *Brettanomyces* and its teleomorph *Dekkera*, causes spoilage aromas associated with “mousiness”, “horsey”, “wet dog” and “medicinal” flavours (Chatonnet *et al*., 1992). These aromas result from high concentrations of acetic acid, as well as 4-ethylphenol, which is an indicator of the presence of *Brettanomyces* species (Chatonnet *et al*., 1992; Fugelsang, 1997). This compound is only considered to be a spoilage factor when it is present in the wine at concentrations exceeding 620 $\mu g.L^{-1}$. At lower concentrations (< 400 $\mu g.L^{-1}$) it contributes favorably to the wine by giving it a “spicy”, “smokey” or “leathery” flavour (Chatonnet *et al*., 1992; Loureiro & Malfeito-Ferreira, 2003). *Brettanomyces intermedius* and *Brettanomyces anomalus* can also produce significant quantities of 4-ethylguaiacol. This compound gives a pleasant “clove-like” or “spicy” odour to the wine at low concentrations (Fugelsang, 1997).

Of the non-*Saccharomyces* yeasts, *Hanseniaspora uvarum* represents the dominant yeast species present on the grapes. *Hanseniaspora uvarum* and *Hanseniaspora apiculata* can produce acetic acid and esters in the early stages of alcoholic fermentation which contribute to the volatile aroma of the fermenting wine (Fugelsang, 1997). Romano *et al*. (2003) found that *H. uvarum* produced high concentrations of acetoin and ethyl acetate in the early stages of fermentation, while a low production of higher alcohols occurred. *Candida stellata* also produced high levels of acetoin and ethyl acetate early in the fermentation process, with a low production of higher alcohols.

Zygosaccharomyces species isolated from grape must and fermenting wine include *Zygosaccharomyces bailii*, *Zygosaccharomyces bisporous*, *Zygosaccharomyces fermentati*, *Zygosaccharomyces florentinus* and *Zygosaccharomyces rouxii* (Fugelsang, 1997; Romano *et al*., 2003). *Zygosaccharomyces* spp. favour high sugar concentrations

and some strains are particularly resistant to high concentrations of alcohol (>10% (v/v)) and preservatives such as SO_2. However, phenols and anthocyanins in the wine can be inhibitory to *Zygosaccharomyces* spp. (Fugelsang, 1997). Of these yeasts, *Z. bailii* has been identified to cause spoilage most often by the production of high concentrations of acetic acid and esters. Furthermore, *Z. bailii* causes turbidity and sediment in the wine, as well as the reduction of acidity by the metabolism of L-malic acid (Sponholz, 1993).

Other non-*Saccharomyces* spoilage yeasts isolated from wine include *Pichia* spp., *Saccharomycodes ludwigii* and *Schizosaccahromyces pombe*. *Pichia membraefaciens*, *Pichia vini* and *Pichia farinose* are usually found during the early stages of fermentation, since inhibition occurs at alcohol levels approaching 10% (v/v) (Fugelsang, 1997). These yeast species produce a chalky film on the wine, as well as high concentrations of acetaldehyde (Fleet, 1993). *Saccharomycodes ludwigii* produces high concentrations of acetoin, acetaldehyde, ethyl acetate, and higher alcohols, such as isoamyl alcohol and isobutanol (Fugelsang, 1997; Romano *et al*., 2003). It is resistant to sorbic acid and SO_2, but growth is seldom found in cellars and bottled wine (Fugelsang, 1997). *Schizosaccharomyces pombe* can act as a substitute for LAB by increasing the pH of grape must and by converting L-malic acid to ethanol. It can tolerate ethanol concentrations higher than 10% (v/v) and has been isolated from bottled wine (Boulton *et al*., 1996; Fugelsang, 1997; Fleet, 2003).

The yeast population on the grapes at harvest is influenced by the grape variety, temperature and environmental factors, ripeness and physical damage of the grapes, fungicide use, as well as the methods used to harvest and transport the grapes (Fleet & Heard, 1993; Lambrechts & Pretorius, 2000). Therefore, the organopleptic quality of the wine may vary significantly with the varying microbial content of the grapes (Lambrechts & Pretorius, 2000).

When the grapes undergo stress resulting from bruising or cutting of the skin and flesh either by moulds, insects or birds the microorganism population will increase (Fleet & Heard, 1993; Fleet, 2003). Such damaged grapes in the vineyard show increased numbers of *Hanseniaspora* and *Metschnikowia* spp., which are the predominating species of non-*Saccharomyces* yeasts on grapes, as well as species of *Aureobasidium*,

Candida, *Saccharomyces* and *Zygosaccharomyces* (Fleet, 2003; Prakitchaiwattana *et al*., 2004).

Saccharomyces cerevisiae

Saccharomyces cerevisiae is rarely isolated from grape berries, therefore, to encourage the fermentation and improve control over the process many winemakers add specialised strains of *S. cerevisiae* to the grape must (Fugelsang, 1997; Querol *et al*., 2003). These strains are chosen according to their ability to ferment glucose and fructose present in the grape must, their ethanol tolerance and production, dynamic fermentation action, tolerance of different temperatures and high sugar environments, glycerol production, resistance to SO_2, as well as the production of foam, H_2S, volatile acidity and acetaldehyde (Pérez-Coello *et al*., 1999; Esteve-Zarzoso *et al*., 2000). The fermentation is completed more rapidly with inoculated grape juice and the wines produced are of reliable quality. However, by inoculating the must with high numbers of *S. cerevisiae* does not inhibit the growth of the non-*Saccharomyces* yeasts, and the fermentation would not automatically be dominated by the inoculum (Fleet & Heard, 1993).

Different *S. cerevisiae* strains may occur during the fermentation of grape must, all producing different levels of desirable or undesirable secondary metabolites, such as varying levels of higher alcohols, acetaldehyde and acetic acid. *S. cerevisiae* produces high concentrations of the higher alcohols isoamyl alcohol and 2,3-butanediol, while a lower production of acetoin is observed. Acetic acid production below the threshold level of 600 $mg.L^{-1}$ occurs in all known *S. cerevisiae* strains (Romano *et al*., 2003).

Factors affecting the growth of yeasts during wine fermentation

The addition of SO_2 to grape must restricts the growth of spoilage organisms. The efficacy of SO_2 preservation varies with the concentration of the added SO_2, as well as the composition of the grape juice and the SO_2 tolerance of the microorganisms present in the must (Fleet & Heard, 1993). It is a general assumption that SO_2 addition to grape musts will selectively limit the growth of the non-*Saccharomyces* yeast species present

on the grapes and, therefore, promote the growth of *S. cerevisiae*. However, since the presence of *Kloeckera* and *Candida* species have been found in commercial wine fermentations even after the addition of the standard 50 - 100 $mg.L^{-1}$ SO_2, the effectiveness of SO_2 in controlling the yeast species are controversial (Fleet & Heard, 1993).

Another factor influencing the fermentation process is a stuck or sluggish fermentation, during which yeasts die-off too early resulting in a product that is high in unfermented sugar with a low ethanol concentration. It is generally caused by factors such as temperature and nutrient reduction that will affect yeast growth (Fleet & Heard, 1993). A sluggish fermentation can also be caused when the dry commercial yeast are not re-hydrated at an appropriate temperature (37° - 40°C), and appropriately cooled down. This is essential before adding the inoculum to the grape must, since it can influence the viability of the yeast cells by up to 60% (Fugelsang, 1997).

While the grape must is fermenting, the fermentation temperature influences the growth-rate of the yeasts. This in turn affects the duration of the fermentation process, the metabolic contributions of the various yeast species to the fermentation and the biochemical reactions of the yeasts, which will in turn influence the wine quality. The different yeast species grow at different optimum temperatures and their ethanol tolerance is affected by the temperature of the fermentation. The non-*Saccharomyces* yeasts can tolerate higher ethanol concentrations at lower temperatures. This can lead to a possible dominance of the alcoholic fermentation process by these yeasts (Fleet & Heard, 1993).

The presence of other microorganisms, such as killer yeasts, mycelial fungi, LAB and AAB can inhibit the growth of yeasts. They can either deplete the available nutrients, especially where the alcoholic fermentation initiation is slow, or produce compounds such as toxins and acetic acid that is inhibitory to yeasts (Fleet & Heard, 1993; Fleet, 2003).

Lactic acid bacteria

MLF is the secondary fermentation step after alcoholic fermentation in wine (Boulton *et al*., 1996; Alexandre *et al*., 2004), and refers to the conversion of L(-) malic acid, one of the most common acids in grape must and wine to L(+) lactic acid by LAB (Fig. 1), with the requirements of NAD^+ and Mn^{2+} and the production of CO_2 as a metabolic by-product. The completion of MLF results in the depletion of malic acid and the resultant microbial stability (Boulton *et al*., 1996).

The LAB responsible for MLF in wine are members of the *Lactobacillaceae*, characterised by the genus *Lactobacilllus*, and the *Streptococcaceae*, characterised by the genera *Pediococcus* and *Leuconostoc* (Henick-Kling, 1993; Fugelsang, 1997). *Pediococcus damnosus*, *Leuconostoc mesenteroides* and *O. oeni* [previously *Leuconostoc oenos* (Lonvaud-Funel, 1999)] has been identified as the key LAB accountable for MLF (Lonvaud-Funel, 1999). However, only *O. oeni* have been shown to tolerate the conditions of wine and are, therefore, frequently used as a starter culture for MLF (Lonvaud-Funel *et al*., 1991; Van Vuuren & Dicks, 1993; Fugelsang, 1997). These LAB can be either homofermentative such as *Pediococcus* spp., or heterofermentative such as *O. oeni*, while the *Lactobacilli* may be found in both groups. Homofermentative microbes convert the glucose to lactic acid via the Embden-Meyerhof pathway (Fig. 2) (Fugelsang, 1997). Heterofermentative bacteria make use of the 6-phosphogluconate pathway to produce lactic acid, as well as ethanol, acetic acid and CO_2 (Fig. 3). The metabolic end-products of these two pathways of sugar utilisation can initially be used to determine which LAB are present in the wine (Lonvaud-Funel, 1995; Fugelsang, 1997).

Factors affecting the growth of LAB

During MLF various interactions can occur between the wine yeasts and the LAB that can affect the ability of the LAB to successfully complete MLF. Such interactions can either be favourable or unfavorable for the growth of LAB (Fugelsang, 1997; Fleet, 2003;

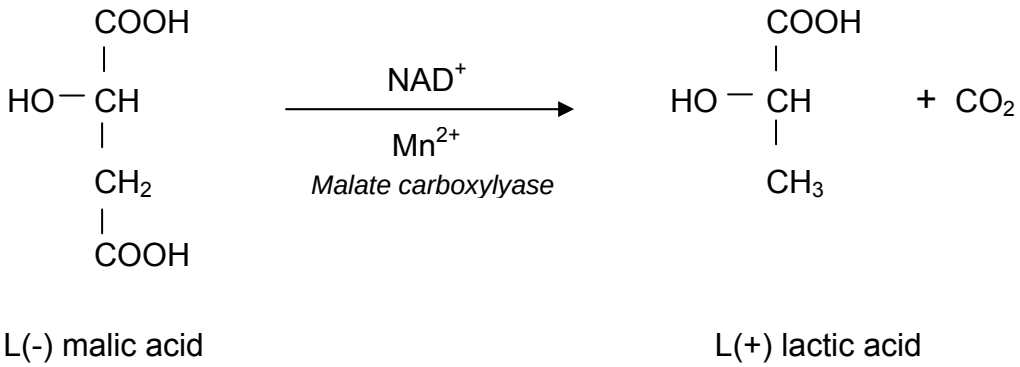


Figure 1 The malo-lactic conversion of L(-) malic acid to L(+) lactic acid by lactic acid bacteria (LAB) during malo-lactic fermentation (MLF) (Boulton *et al*., 1996).

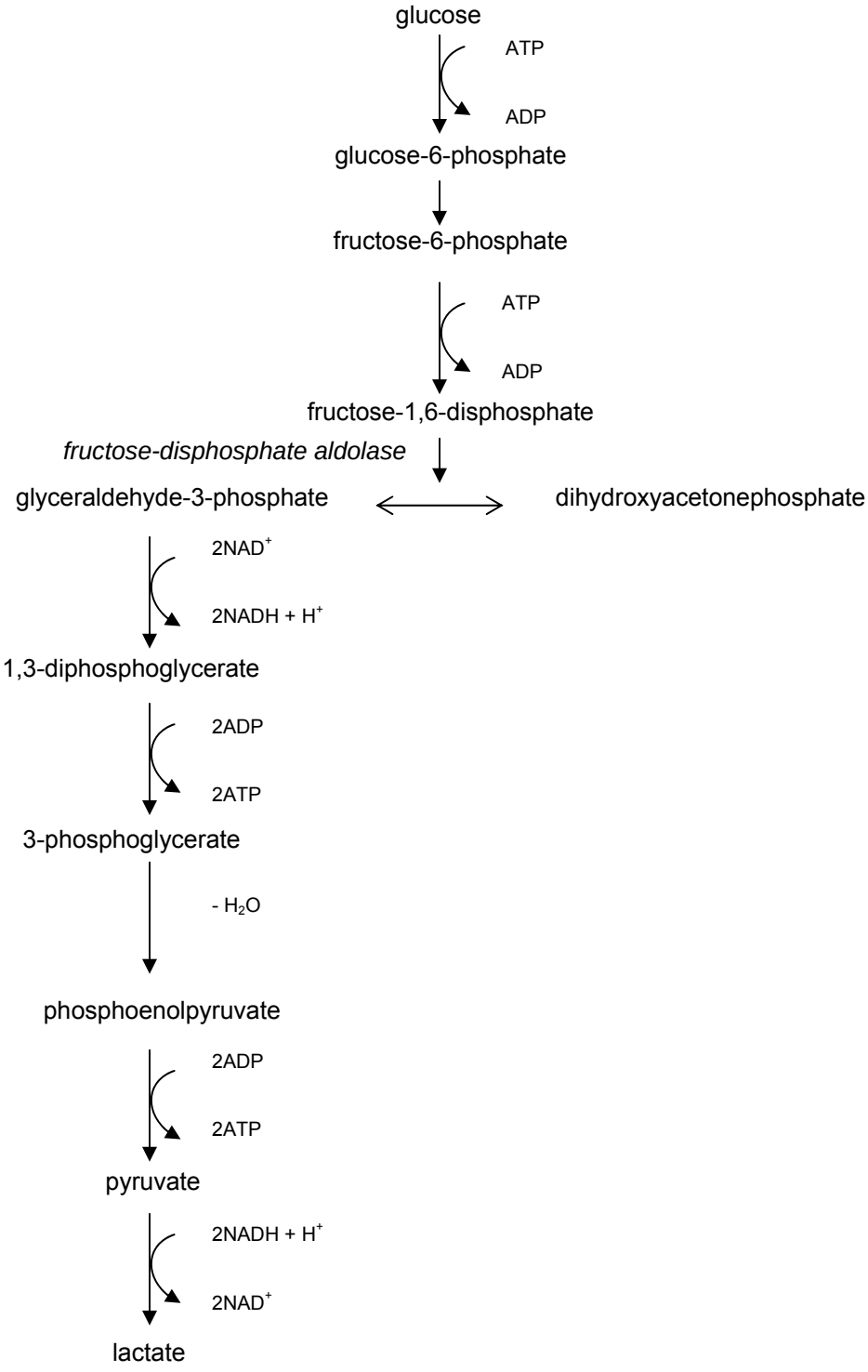


Figure 2 Embden-Meyerhof pathway for the conversion of glucose to lactic acid by homofermentative lactic acid bacteria (LAB) (Boulton *et al*., 1996; Fugelsang, 1997).

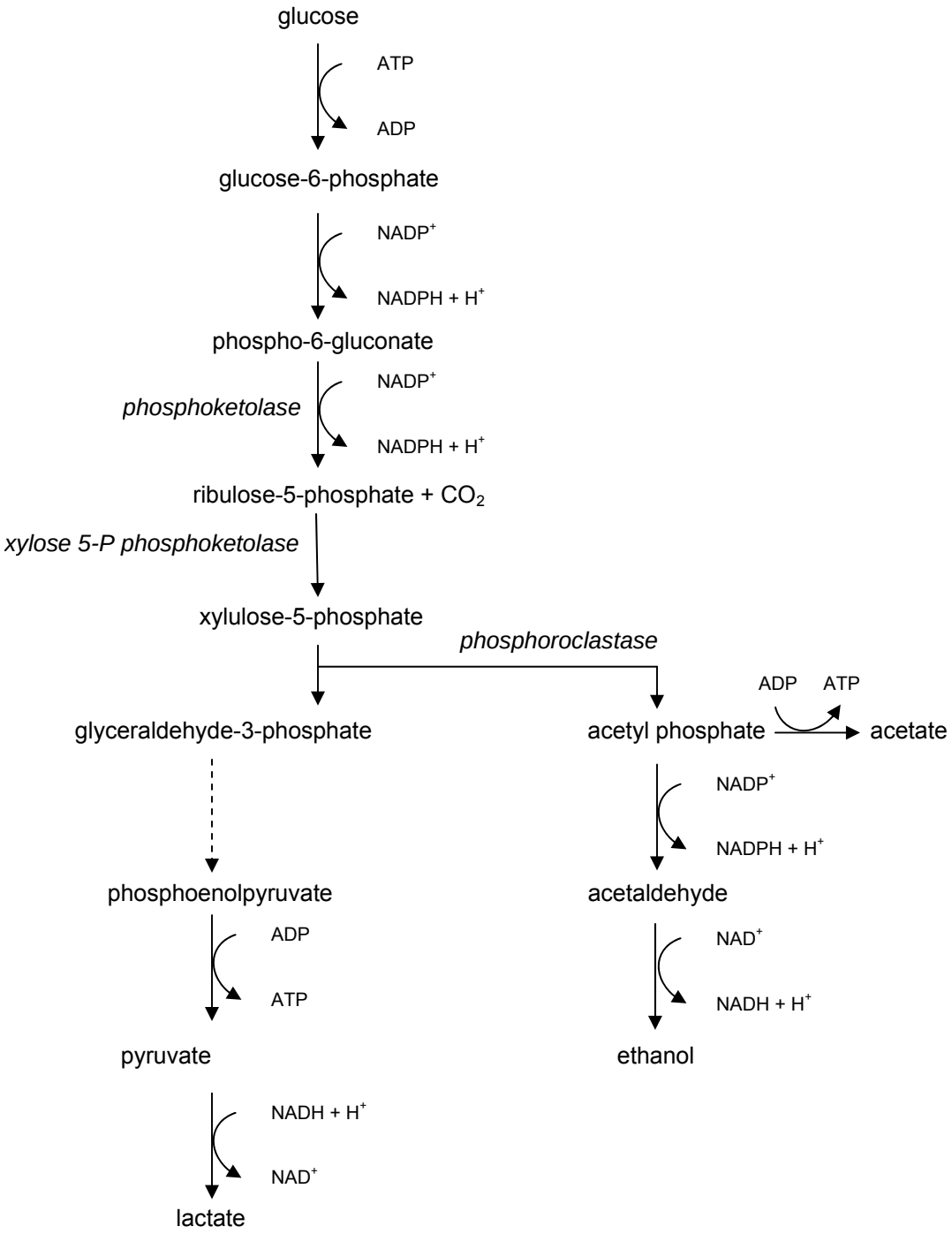


Figure 3 6-Phosphogluconate pathway for the conversion of glucose to lactic acid by heterofermentative lactic acid bacteria (LAB) (Boulton *et al*., 1996; Fugelsang, 1997).

Alexandre *et al*., 2004). In addition to the interactions between the microbes, their metabolic products also affect the LAB, such as high ethanol levels produced by yeasts (Bauer & Dicks, 2004). *Lactobacillus plantarum* die-off between 5 - 6% (v/v) ethanol, while *L. casei* and *L. brevis* can endure higher ethanol concentrations (Henick-Kling, 1993). However, the pH of the medium has an effect on the ethanol tolerance, where higher pH levels (pH 5.0) can increase the ethanol tolerance of *O. oeni* (Capucho & San Romano, 1994). The growth of *O. oeni* is partially inhibited by ethanol concentrations above 5% (v/v), but can endure ethanol concentrations of up to 14% (v/v) (Henick-Kling, 1993; Capucho & San Romano, 1994). It favours low pH conditions and, therefore, prevail in wine with pH values below 3.5, while *Lactobacillus* and *Pediococcus* spp. are associated with wines at higher pH levels (pH > 3.5) (Henick-Kling, 1993). It has also been noted that high fermentation temperatures reduce the ability of LAB to tolerate ethanol. Optimal growth conditions for *O. oeni*, is a fermentation temperature of *ca* 30°C with an ethanol concentration of between 0 - 4% (v/v). When the fermentation temperature is decreased (18° - 25°C) the LAB can tolerate higher ethanol concentrations (10 - 14% (v/v) (Henick-Kling, 1993).

LAB are also inhibited by SO_2, produced by wine yeasts as a metabolic by-product. SO_2 can be added at the start of fermentation to prevent spoilage but may, in high concentrations hinder the onset of MLF (Fleet, 2003; Alexandre *et al*., 2004). Although the current understanding of the influence of SO_2 on LAB is limited, it is known that 100 – 150 $mg.L^{-1}$ total SO_2 inhibits these microbes. The tolerance of LAB to SO_2 is also dependant on the species of LAB present in the wine, where *Lactobacillus* and *Pediococcus* spp. are more tolerant to higher concentrations (Romano & Suzzi, 1993).

Another growth inhibitor of LAB are medium chain fatty acids, especially decanoic acid (Lafon-Lafourcade *et al*., 1984; Edwards & Beelman, 1987). This fatty acid prevents the ability of LAB to metabolise malic acid at concentrations of 5 – 10 $mg.L^{-1}$ (Edwards & Beelman, 1987). The pH also plays a critical role in the inhibitory effect of these fatty acids and at pH > 6.0 they do not have such an inhibitory effect on MLF than at a pH of 3.0 (Capucho & San Romano, 1994). It has been reported by Capucho and San Romano (1994) that very low concentrations

of decanoic acid anddodecanoic acid (12.5 mg.L^{-1} and 2.5 mg.L^{-1}, respectively) can stimulate malic acid degradation. Furthermore, the combination of various medium chained fatty acids, such as hexanoic, octanoic and decanoic acids can lead to greater inhibition of bacterial growth (Lonvaud-Funel *et al*., 1988).

Spoilage caused by LAB

Although LAB play an important role in improving the organoleptic quality of the wine, these microbes can also cause spoilage. This includes the production of D-lactic acid, acetic acid, diacetyl, tartaric acid degradation, “mousiness”, “bitterness”, geranium off-tone and ropiness (Sponholz, 1993). The presence of D-lactic acid, together with mannitol and acetic acid produced by the LAB give a “vinegary” flavour to the wine. Furthermore, the heterofermentative LAB, *Lactobacillus* and *Leuconostoc*, produces D-lactic and acetic acids which can lead to acidification of the wine (Sponholz, 1993). The acetic acid produced by the heterofermentative LAB is different in organoleptic quality to the acetic acid produced by AAB. The acetic acid produced by AAB contains ethyl acetate in the metabolic consortium, while this compound is either absent or present in very low concentrations from LAB metabolism, which makes the acetic acid less detectable even when it is present at concentrations exceeding legal limits (Fugelsang, 1997).

Another spoilage condition caused by LAB in wine is high levels of diacetyl resulting in a characteristic “buttery” off-flavour at high concentrations (> 5 mg.L^{-1}) (Henick-Kling, 1993). It is formed by the metabolism of citric acid, with acetoin and acetic acid as by-products (Fugelsang, 1997). This compound can be produced by yeasts at concentrations of 0.2 - 0.3 mg.L^{-1} (Fugelsang, 1997). However, the majority of diacetyl is produced by LAB during MLF (Sponholz, 1993). At low concentrations (1 – 4 mg.L^{-1}) this compound can enhance the organopleptic quality of the wine. The levels of diacetyl production may vary, with *O*. *oeni* producing low concentrations of diacetyl, while *Lactobacillus* and *Pediococcus* spp. generally produces higher concentrations of this compound (Sponholz, 1993; Fugelsang, 1997).

Tartaric acid is not readily broken down by microbes present in the wine and by the time of its metabolism, the wine will have already been spoilt by other

factors. Only a small number of *Lactobacillus* spp. can metabolise tartaric acid, including *Lactobacillus plantarum* and *Lactobacillus brevis*. *Lactobacillus plantarum* metabolises tartaric acid to oxaloacetate and then to CO_2 and pyruvic acid, which in turn is then reduced to either lactic acid or acetic acid and CO_2. *Lactobacillus brevis* metabolises tartaric acid to oxaloacetic acid and then to either succinic acid or acetic acid and CO_2. The increase in concentrations of these compounds will result in spoilage (Sponholz, 1993).

Although not a frequent problem, the “mousiness” defect arise in low acid wines containing no or low concentrations of SO_2 (Sponholz, 1993). The heterofermentative LAB, such as *L. brevis*, *Lactobacillus hilgardii and Lactobacillus cellobiosus* are able to produce 2-acetyl-1,4,5,6-tetrahydropyridine and its isomer, 2-acetyl-3,4,5,6-tetrahydropyridine, which gives a damp, mousy tone to the wine. Since ethanol is required as a substrate to form these metabolites, the “mousiness” defect is largely present in the wine, and not in the grape must (Sponholz, 1993; Fugelsang, 1997).

“Bitterness”, a well-known defect in wine, is a result of glycerol catabolism in red wine (Sponholz, 1993). Glycerol is an essential element of wine and is produced by yeasts during alcoholic fermentation. The concentrations of glycerol can reach 3 - 14 $g.L^{-1}$ (Drysdale & Fleet, 1988). When the glycerol is degraded, 3-hydroxypropionaldehyde is produced which is further dehydrated to acrolein by a dehydratase enzyme or spontaneously by heat. The acrolein then reacts with the phenolic groups of anthocyanins in the wine and is detectable at acrolein concentrations as low as *ca* 10 parts per million (ppm). The ability to degrade glycerol is not common amongst LAB, where only 31% of *Lactobacillus* spp. have shown to have this ability (Sponholz, 1993).

Geranium off-tone can occur when sorbic acid is used as a preservative against wild yeasts. At the standard application of 200 $mg.L^{-1}$, it is not effective against LAB. Certain LAB (*O. oeni* and some *Lactobacillus* spp.*)* can metabolise the sorbic acid to sorbinol, which isomerises and reacts with alcohol to form 2-ethoxyhexa-3,5-diene. This compound is responsible for the off-odour comparable with crushed geranium leaves (Sponholz, 1993; Fugelsang, 1997).

The “ropiness” defect in wine results from the formation of extracellular dextrins. It is detected by a slimy, “oily” character of the wine, as well as an increase in the wine viscosity. This defect generally occurs in low-acid wines caused by the growth of *Pediocccus* spp., although *Leuconstoc* spp. have also been associated with the defect in sweet wines (Sponholz, 1993, Fugelsang, 1997).

Other spoilage compounds include biogenic amines such as histamine, tyramine and putrescine which are produced by LAB through the decarboxylation of amino acids. At too high concentrations, these biogenic amines can result in adverse physiological effects in humans, such as headaches, respiratory distress, allergic reactions, heart palpitations and hyper- or hypotension (Silla Santos, 1996; Lonvaud-Funel, 2001).

Acetic acid bacteria

AAB are regarded as spoilage microbes and can metabolise ethanol to acetic acid and acetaldehyde, referred to as the volatile acidity of wine (Drysdale & Fleet, 1988; Fugelsang, 1997). Little research has been done on the effect AAB has on wine quality (Du Toit & Lambrechts, 2002). This may be due to the fact that it was long believed that AAB are obligate aerobes and that growth was not possible given the anaerobic conditions of the winemaking process. It has, however, been shown that certain species of AAB can continue to grow during alcoholic fermentation, MLF and the maturation of the wine (Joyeux *et al*., 1984; Drysdale & Fleet ,1988).

The AAB that are associated with grapes and wine are *Gluconobacter oxydans, Acetobacter aceti*, *Acetobacter pasteurianus, Gluconacetobacter liquefaciens and Gluconacetobacter hansenii* (Du Toit & Lambrechts, 2002). Healthy grapes and the produced must are associated with *G. oxydans*, which favor high sugar concentrations and is more common at the beginning stages of fermentation. As the alcohol concentration increases during the later stages of fermentation, this microbe dies-off (Joyeux *et al*., 1984). In a study conducted by Drysdale and Fleet (1989), *G. oxydans* was unable to grow or survive in fermented

wine, even when the wine was aerated. On the other hand, *Acetobacter* spp. favours ethanol (De Ley *et al*., 1984) and are more often isolated during the latter stages of alcoholic fermentation (Joyeux *et al*., 1984).

Factors affecting the growth of AAB

Factors which significantly affect the growth of AAB in wine are the pH, ethanol concentration, temperature, oxygen level and SO_2 concentration (Drysdale & Fleet, 1988). Although the optimum pH range for AAB is between 5.4 and 6.3 (De Ley *et al*., 1984), it has been reported that AAB are tolerant of the low pH of wine (3.0 - 4.0) (Drysdale & Fleet, 1988). Joyeux *et al*. (1984) has shown reduced growth of *A*. *aceti* at pH 3.4 compared to growth at pH 3.8. In the same study, it was shown that a temperature of 10°C reduced the growth of *A*. *aceti*, while at 18°C the cell numbers increased 30 - 40 fold. However, the optimum temperature range for the growth of AAB is between 25° – 30°C (De Ley & Swings, 1984; De Ley *et al*., 1984).

Apart from the pH and temperature, the ethanol concentration also affects the growth of AAB. Only some *Gluconobacter* spp. can grow in ethanol concentrations of 5% (v/v) (De Ley & Swings, 1984), while *Acetobacter* spp. have been isolated from wine, indicating that they can tolerate concentrations of 10 - 15% (v/v) ethanol (Drysdale & Fleet, 1988).

Although the winemaking processes appear to be anaerobic, oxygen can access the wine through the pumping or transferring of the wine to the barrels which can lead to increased growth of AAB (Joyeux *et al*., 1984; Drysdale & Fleet, 1989). Drysdale and Fleet (1989) have shown that *A. pasteurianus* and *A. aceti* can survive in stored wine, since they remain viable at oxygen concentrations of as low as *ca* 30% (v/v). Joyeux *et al*. (1984) confirmed this, especially in wine that is stored in wooden barrels, since oxygen can be transferred through the wood into the wine at concentrations of up to 30 $mg.L^{-1}$ per year. In these low-oxygen environments, the AAB can also utilise electron acceptors other than oxygen, such as phenolics in their metabolic cycle (Fugelsang, 1997).

Sensory implications of AAB spoilage

AAB can raise the level of acetic acid in the wine to unacceptable levels, known as the acetification of wine (Drysdale & Fleet, 1988). The legal limit for acid in wine is 1200 – 1300 $mg.L^{-1}$, although it has been reported to cause spoilage at >700 $mg.L^{-1}$ (Fugelsang, 1997).

Apart from producing acetic acid, AAB also produce acetaldehyde, gluconate and ketogluconate that affect the wine quality unfavourably (Drysdale & Fleet, 1988). Of these compounds, acetaldehyde is present in wine through the metabolism of yeasts (Fugelsang, 1997). This compound is also produced by *A. pasteurianus* and *A. aceti* in the presence of low oxygen concentrations. The reduced oxygen environment can lead to the increased functioning of ethanol dehydrogenase and a decrease in the action of acetaldehyde dehydrogenase, leading to an accumulation of acetaldehyde in the wine. Consequently, too high concentrations of acetaldehyde gives the wine an oxidized colour and flavour (Drysdale & Fleet, 1989) and is organoleptically perceived as “nutty”, “sherry-like” or even “bruised apples” with an aroma threshold of 100 - 120 $mg.L^{-1}$ (Fugelsang, 1997).

The volatile acidity of the wine is not only the result of acetic acid, but is also due to the presence of various acetate esters, especially ethyl acetate. This compound is sensorially perceived as “aeroplane glue” or “nail polish” at concentrations as low as 12.3 $mg.L^{-1}$ (Fugelsang, 1997). Wines that are affected can contain concentrations of 150 – 200 $mg.L^{-1}$ ethyl acetate (Boulton *et al*., 1996).

Although glycerol is a natural product of yeast metabolism, too high concentrations can be found in the grape must if the grape berries are infected with the fungus *Botrytis cinerea*. This creates the perfect opportunity for *G. oxydans* and *A. aceti* to oxidize glycerol to dihydroxyacetone, which affects the sensory quality of the wine. This compound gives a sweet bouquet and a cooling flavour to the wine and combining it with certain amino acids, such as proline creates a strong “crusty” odour (Fugelsang, 1997).

Acetoin is another spoilage compound produced by AAB by the utilization of lactic acid under low oxygen concentrations. It gives the wine a “buttery” flavour

when present at levels of 3 – 31.8 $mg.L^{-1}$ (Drysdale & Fleet, 1988, Fugelsang, 1997).

D. Methods for detection and identification of microbes present in wine

Different organoleptic and quality characteristics, as well as spoilage conditions in wine is caused by the metabolism of yeasts, LAB or AAB and the interactions between them. Methods used for the detection and identification of these microbes in must and wine includes conventional microbiological plating methods and molecular approaches. Conventional microbiological plating methods, involving selective cultivation and isolation of microbes based on their physiological abilities are commonly used to identify the different yeast and bacterial species present in wine (Fleet & Heard, 1993; Dias *et al*., 2003; Medawar *et al*., 2003; Pasteris & Strasser de Saad, 2005; Ciani *et al*., 2006; Pérez-Nevado *et al*., 2006; Renouf & Lonvaud-Funel, 2006), to differentiate between homofermentative and heterofermentative LAB (Zúñiga *et al*., 1993), and to study the malo-lactic activity of starter LAB (Krieger *et al*., 1992). Microbiological plating has also been used to identify spoilage yeasts, LAB and AAB (Ubeda & Briones, 1999). However, these plating methods are time-consuming and not all microbes can be cultured on synthetic growth media (Heard & Fleet, 1986; Kopke *et al*., 2000).

Molecular techniques have proven to be reliable and rapid alternatives for conventional microbiological plating (Ercolini, 2004). Several molecular-based methods have been developed to detect and identify microbes present during wine fermentations, such as polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) (Cocolin *et al*., 2000; Prakitchaiwattana *et al*., 2004), PCR-based temperature gradient gel electrophoresis (TGGE) (Fernández-González *et al*., 2001), fluorescence *in situ* hybridisation (FISH) (Xufre *et al*., 2006), restriction fragment length polymorphism (RFLP) (Guillamón *et al*., 1998; Fernández *et al*., 1999; Granchi *et al*., 1999) and amplified fragment length polymorphism (AFLP) (Gallego *et al*., 2005).

PCR-based DGGE is a commonly used method for the culture–independent fingerprinting of the microbial diversity in various environmental samples (Ercolini, 2004). It is a method based on extracting DNA from samples and amplifying the DNA by PCR with specific primers, which is followed by electrophoresis with the separation and detection of PCR-amplified products of the same length, but different base pair composition (Fig. 4). This is accomplished by the electrophorectic mobility and the different melting profiles of the different fragments in a polyacrylamide gel (Muyzer *et* al., 1993; Ercolini, 2004). DGGE of PCR-amplified fragments has emerged as a powerful diagnostic tool for the direct profiling of the microbial diversity present in wine without the need for cultivation (Cocolin *et al*., 2000; Kawai *et al*., 2002; Lopez *et al*., 2003; Prakitchaiwattana *et al*., 2004).

Similarly to PCR-DGGE, PCR-TGGE is based on gradual and uniform increase in the temperature during electrophoresis and a denaturing environment is formed by the urea in the gel (Muyzer, 1999) and has been used by Fernández-González *et al*. (2001) to profile yeasts during wine fermentation. Using PCR-TGGE they detected *Saccharomyces*, *Kluyveromyces*, *Hanseniaspora*, *Candida*, and *Rhodotorula* in the wine samples, compared to only *Kluyveromyces*, *Candida*, *Saccharomyces* and *Hanseniaspora* isolated from plating methods.

Both these techniques are rapid (Fernández-González *et al*., 2001; Ercolini, 2004) and verify culture purity (Hernán-Gómez *et al*., 2000). Samples can be directly analysed without prior enrichment and cultivation (Ercolini, 2004). However, these techniques also have disadvantages. To ensure proper amplification and separation of the fragments, high yields of DNA of all the species present are required. Difficulty exists to extract DNA from all the species present with the same efficacy (Ercolini, 2004). Also, some wine compounds can inhibit the DNA extraction and PCR reaction and there is a possibility that during PCR amplification, some DNA templates may be inhibited or favoured by the reaction resulting in misleading results (Fernández-González *et al*., 2001; Ercolini, 2004). Furthermore, a single species with multiple ribosomal RNA (rRNA) copies can portray multiple bands in the DGGE profile, overestimating the diversity of the

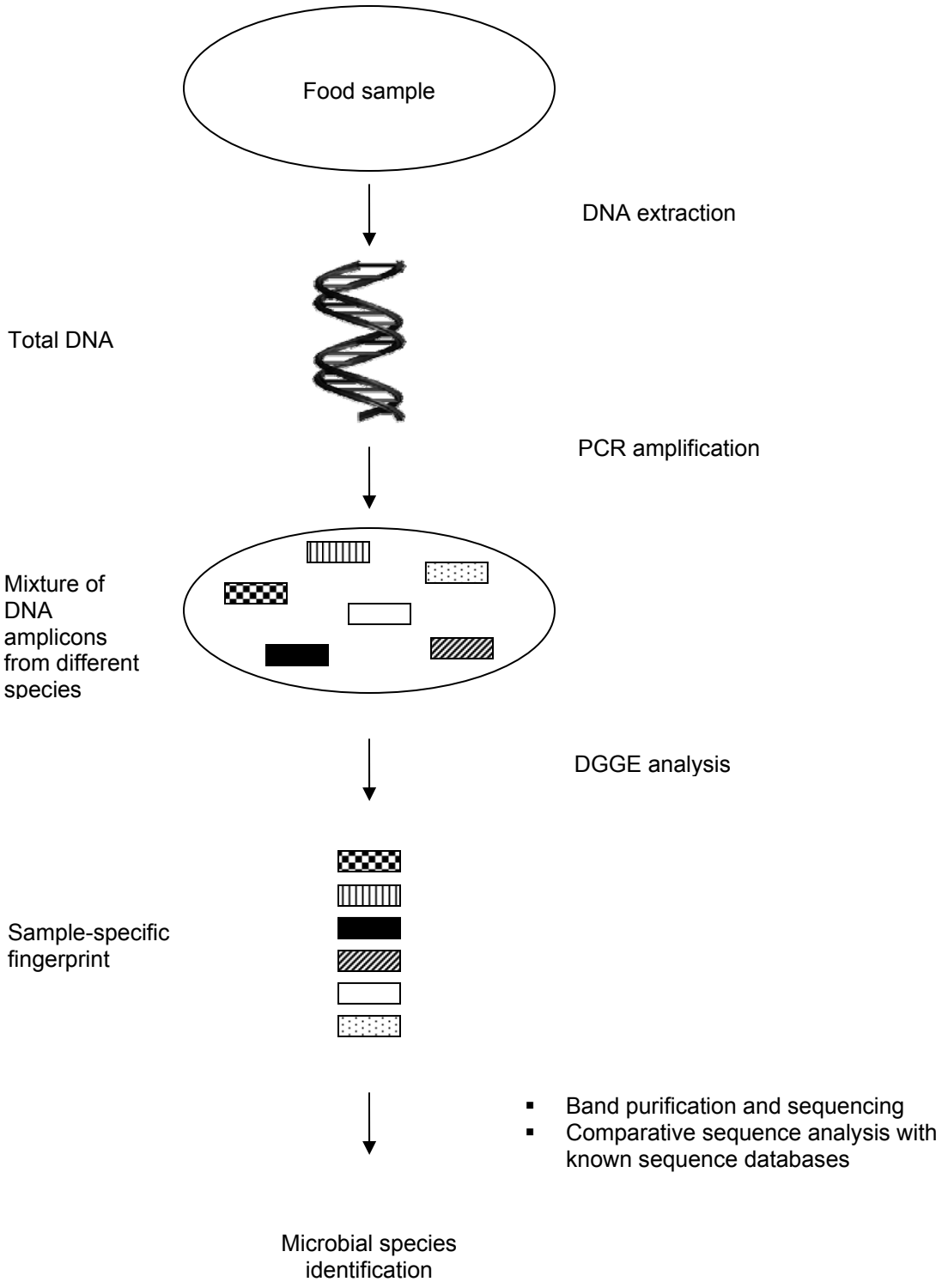


Figure 4 Flow diagram of the application of PCR-based DGGE analysis to food samples (Ercolini, 2004).

microbes in the sample (Nübel *et al*., 1996).

Another culture-independent technique, FISH, combines direct visualisation with molecular approaches (Xufre *et al*., 2006). This method detects nucleic acid sequences by an oligonucleotide probe that is fluorescently labeled and hybridises exclusively to its complementary target sequence within the undamaged cell. A complete three-dimensional view of the diversity and dynamics of the microbes present are obtained. FISH can provide information on the occurrence, quantity, morphology and distribution of microbes in a sample. It is a fast method of detection, since no isolation and cultivation is required (Moter & Göbel, 2000). This technique has been employed by Xufre *et al*. (2006) to analyse the yeast population present in grape must fermentations.

During RFLP analyses the DNA is digested with one or more restriction enzymes and the fragments are then separated by gel electrophoresis. This method has been used by Guillamón *et al*. (1998) and Granchi *et al*. (1999) to identify yeasts from wine fermentations. In both cases it was found that this is a rapid, easy and reproducible method of yeast identification. Fernández *et al*. (1999) compared PCR-RFLP with conventional methods using wine samples and found that RFLP are more discriminatory and can be used to verify or correct identifications from conventional microbiological methods. The same technique was used to successfully identify *O*. *oeni* from red wine samples (Sato *et al*., 2000).

AFLP is a fingerprinting technique based on the restriction of the DNA and ligation of oligonucleotide adapters, selective amplification of the restriction fragments by PCR and gel analysis of the fragments (Vos *et al*., 1995). This method was by used by Gallego *et al*. (2005) to distinguish between different strains of *S. cerevisiae* in wine.

E. Conclusion

Wine fermentation is a complex and biochemical process involving interactions between yeasts, LAB and AAB, as well as their individual metabolisms. These microbes cause desirable or undesirable organoleptic and quality characteristics of

the wine. Consequently, it is important to understand the different microbes and their interactions during and on completion of the fermentation process (Fleet, 2003). Conventional microbiological methods can be used to identify the different microorganisms present in wine, but prove to be time-consuming and certain microbial species do not grow on synthetic growth media, leading to false information of the microbial population present in the wine (Kopke *et al*., 2000). To overcome these problems, culture-independent approaches are used as alternatives to traditional microbiological techniques. Of these methods, PCR-based DGGE has been used successfully in wine analysis, and appears to be the best culture-independent method to identify complete microbial populations (Guillamón *et al*., 1998; Fernández *et al*., 1999; Granchi *et al*., 1999; Cocolin *et al*., 2000; Fernández-González *et al*., 2001; Prakitchaiwattana *et al*., 2004; Gallego *et al*., 2005; Xufre *et al*., 2006).

References

Alexandre, H., Costello, P.J., Remize, F., Guzzo, J. & Guilloux-Benatier, M. (2004). *Saccharomyces cerevisiae-Oenococcus oeni* interactions in wine: current knowledge and perspectives. *International Journal of Food Microbiology*, **93**, 141-154.

Bartowsky, E.J. & Henschke, P.A. (2004). The “buttery” attribute of wine – diacetyl – desirability, spoilage and beyond. *International Journal of Food Microbiology*, **96**, 235 – 252.

Bauer, R. & Dicks, L.M.T. (2004). Control of malo-lactic fermentation in wine. A review. *South African Journal of Enology and Viticulture*, **25**, 274-88.

Boulton, R.B., Singleton, V.L., Bisson, L.F. & Kunkee, R.E. (1996). Principles and practices of winemaking. Pp. 132-133, 245, 247. New York: Chapman & Hall.

Capucho, I. & San Romano, M.V. (1994). Effect of ethanol and fatty acids on malo-lactic activity of *Leuconostoc oenos*. *Applied Microbiology and Biotechnology*, **42**, 391-395.

Chatonnet, P., Dubourdieu, D., Boidron, J.N. & Pons, M. (1992). The origin of ethylphenols in wines. *Journal of Science Food Agriculture*, **60**, 165-178.

Ciani, M., Beco, L. & Comitini, F. (2006). Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *International Journal of Food Microbiology*, **108**, 239-245.

Cocolin, L., Bisson, L.F. & Mills, D.A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiology Letters*, **189**, 81-87.

De Ley, J. & Swings, J. (1984). Genus II. *Gluconobacter Asai* (1935). In: Bergey's Manual of Systematic Bacteriology, Volume 1, 9th ed. Pp 275-284. Baltimore: Williams and Wilkens.

De Ley, J., Swings, J. & Gossele, F. (1984). Genus I. *Acetobacter Beijerinck* (1898). In: Bergey's Manual of Systematic Bacteriology, Volume 1, 9th ed. Pp 268-274. Baltimore: Williams and Wilkens.

Dias, L., Dias, S., Sancho, T., Stender, H., Querol, A., Malfeito-Ferreira, M. & Loureiro, V. (2003). Identification of yeasts isolated from wine-related environments and capable of producing 4-ethylphenol. *Food Microbiology*, **20**, 567–574.

Du Toit, M. & Pretorius, I.S. (2000). Microbial spoilage and preservation of wine: using weapons from nature's own arsenal – a review. *South African Journal of Enology and Viticulture*, **21**, 74-96.

Du Toit, W.J. & Lambrechts, M.G. (2002). The enumeration and identification of acetic acid bacteria from South African red wine fermentations. *International Journal of Food Microbiology*, **74**, 57-64.

Drysdale, G.S. & Fleet, G.H. (1988). Acetic acid bacteria in winemaking: a review. *American Journal of Enology and Viticulture*, **39**, 143-152.

Drysdale, G.S. & Fleet, G.H. (1989). The growth and survival of acetic acid bacteria in wines and different concentrations of oxygen. *American Journal of Enology and Viticulture*, **40**, 99-105.

Edwards, C.G. & Beelman, R.B. (1987). Inhibition of the malo-lactic bacterium Leuconostoc *oenos* (PSU-1) by decanoic acid and subsequent removal of the inhibition by yeast ghosts. *American Journal of Enology and Viticulture*, **38**, 239-242.

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, **56**, 297-314.

Esteve-Zarzoso, B., Gostíncar, A., Bobet, R., Uruburu, F. & Querol, A. (2000). Selection and molecular characterization of wine yeasts isolated from the 'El Penedès" area (Spain). *Food Microbiology*, **17**, 553-562.

Fernández, M.T., Ubeda, J.F. & Briones, A.I. (1999). Comparitive study of non-*Saccharomyces* microflora of musts in fermentation, by physiological and molecular methods. *FEMS Microbiology Letters*, **173**, 223-229.

Fernández-González, M., Espinosa, J.C., Úbeda, J.F. & Briones, A.I. (2001). Yeasts present during wine fermentation: comparative analysis of conventional plating and PCR-TGGE. *Systematic and Applied Microbiology*, **24**, 634-638.

Fleet, G.H. (1993). The microorganisms of winemaking – isolation, enumeration and identification. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 1-26. New York: Taylor & Francis.

Fleet, G.H. (2003). Yeast interactions and wine flavour. *International Journal of Food Microbiology*, **86**, 11-22.

Fleet, G.H. & Heard, G.M. (1993). Yeasts-growth during fermentation. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 27-54. New York: Taylor & Francis.

Fugelsang, K.C. (1997). Wine Microbiology. Pp. 3-47, 48-67, 68-111, 132-137. New York: Chapman & Hall.

Gallego, J.F., Pérez, M.A., Núñez, Y. & Hidalgo, P. (2005). Comparison of RAPDs, AFLPs and SSR markers for the genetic analysis of yeast strains of *Saccharomyces cerevisiae*. *Food Microbiology*, **22**, 561-568.

González, A., Hierro, N., Poblet, M., Mas, A. & Guillamón, J.M. (2005). Application of molecular methods to demonstrate species and strain evolution of acetic acid bacteria population during wine production. *International Journal of Food Microbiology*, **102,** 295-304.

Granchi, L., Bosco, M., Messini, A. & Vincenzini, M. (1999). Rapid detection and quantification of yeasts species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. *Journal of Applied Microbiology*, **87**, 949-956.

Guillamón, J.M., Sabaté, J., Barrio, E., Cano, J. & Querol, A. (1998). Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. *Archives of Microbiology*, **169**, 387-392.

Heard, G.M. & Fleet, G.H. (1986). Evaluation of selective media for enumeration of yeasts during wine fermentation. *Journal of Applied Bacteriology*, **60**, 477-481.

Henick-Kling, T. (1993). Malo-lactic Fermentation. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 289-326. New York: Taylor & Francis.

Hernán-Gómez, S., Espinosa, J.C. & Ubeda, J.F. (2000). Characteriazation of wine yeasts by temperature gradient gel electrophoresis (TGGE). *FEMS Microbiology Letters*, **193**, 45-50.

Jay, J.M. (1998). Modern Food Microbiology, 5th ed. Pp. 131, 165-166, 278. Maryland: Aspen Publishers Inc.

Joyeux, A., Lafon-Lafourcade, S. & Ribèreau-Gayon, P. (1984). Evolution of acetic acid bacteria during fermentation and storage of wine. *Applied and Environmental Microbiology*, **48**, 153-156.

Kawai, M., Matsutera, E., Kanda, H., Yamaguchi, N., Tani, K. & Nasu, M. (2002). 16S Ribosomal DNA-based analysis of bacterial diversity in purified water used in pharmaceutical manufacturing processes by PCR and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, **68**, 699-704.

Kopke, C., Cristovão, A., Prata, A.M., Silva Pereira, C., Figueiredo Marques, J.J. & San Romão, M.V. (2000). Microbiological control of wine. The application of epfiluorescence microscopy method as a rapid technique. *Food Microbiology*, **17**, 257-260.

Krieger, S.A., Hammes, W.P. & Henick-Kling, T. (1992). Effect of medium composition on growth rate, growth yield and malo-lactic activity of *Leuconostoc oenos* LoZH$_1$-t$_7$-1. *Food Microbiology*, **9**, 1-11.

Lafon-Lafourcade, S., Geneix, C. & Ribéreau-Gayon, P. (1984). Inhibition of alcoholic fermentation of grape must by fatty acids produced by yeasts and their elimination by yeast ghosts. *Applied and Environmental Microbiology*, **47**, 1246-1249.

Lambrechts, M.G. & Pretorius, I.S. (2000). Yeast and its importance to wine aroma – a review. *South African Journal of Enology and Viticulture*, **21**, 97-129.

Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., VanderGheynst, J. & Mills, D.A. (2003). Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, **69**, 6801-6807.

Lonvaud-Funel, A., Joyeux, A. & Dessens, C. (1988). Inhibition of malo-lactic fermentation of wines by products of yeast metabolism. *Journal of the Science of Food and Agriculture*, **44**, 183-191.

Lonvaud-Funel, A., Joyeux, A. & Ledoux, O. (1991). Specific enumeration of lactic acid bacteria in fermenting grape must and wine by colony hybridization with non-isotopic DNA probes. *Journal of Applied Bacteriology*, **71**, 501-508.

Lonvaud-Funel, A. (1995). Microbiology of the malo-lactic fermentation: molecular aspects. *FEMS Microbiology Letters*, **126**, 209-214.

Lonvaud-Funel, A. (1999). Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoek*, **76**, 317-331.

Lonvaud-Funel, A. (2001). Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiology Letters*, **199**, 9-13.

Loureiro, V. & Malfeito-Ferreira, M. (2003). Spoilage yeasts in the wine industry. *International Journal of Food Microbiology*, **86**, 23-50.

Medawar, W., Strehaiano, P. & Délia, M. (2003). Yeast growth: lag phase modeling in alcoholic media. *Food Microbiology*, **20**, 527–532.

Moter, A. & Göbel, U.B. (2000). Fluorescence in situ hybridization (FISH) of direct visualization of microorganisms. *Journal of Microbiological Methods*, **41**, 85-112.

Muyzer, G., de Waal, E.C. & Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, **59**, 695-700.

Muyzer, G. (1999). DGGE/TGGE a method for identifying genes from natural ecosystems. *Current Opinion in Microbiology*, **2**, 317-322.

Nikolaou, E., Soufleros, E.H., Bouloumpasi, E. & Tzanetakis, N. (2005). Selection of indigenous *Saccharomyces cerevisiae* strains according to their oenological characteristics and vinification results. *Food Microbiology*, **23**, 205-211.

Nübel, U., Engelen, B., Fleske, A., Snadir, J., Wieshuber, A., Amann, R., Ludwig, W. & Backhaus, H. (1996). Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology*, **178**, 5636-5643.

Pasteris, S.E. & Strasser de Saad, A.M. (2005). Aerobic glycerol catabolism by *Pediococcus pentosaceus* isolated from wine. *Food Microbiology*, **22**, 399–407.

Pérez-Nevado, F., Albergaria, H., Hogg, T. & Girio F. (2006). Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. *International Journal of Food Microbiology*, **108**, 336–345.

Plata, C., Millán, C., Mauricio, J.C. & Ortega, J.M. (2003). Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. *Food Microbiology*, **20**, 217-224.

Prakitchaiwattana, C.J., Fleet, G.H. & Heard, G.M. (2004). Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Research*, **4**, 865-877.

Querol, A., Fernández-Espinar, M.T., lí del Olmo, M. & Barrio, E. (2003). Adaptive evolution of wine yeast. *International Journal of Food Microbiology*, **86**, 3-10.

Radler, F. (1993). Wine Spoilage by Microorganisms. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 165-182. New York: Taylor & Francis.

Rapp, A. & Mandery, H. (1986). Wine aroma. *Experentia*, **42**, 873-884.

Rapp, A. & Versini, G. (1991). Influence of nitrogen compounds in grapes on aroma compounds in wine. In: *Proceedings of the International symposium on nitrogen in grapes and wines* (edited by RANTZ). Pp. 156-164. Davis, CA: American Society for Enology and Viticulture.

Renouf, V. & Lonvaud-Funel, A. (2006). Development of an enrichment medium to detect *Dekkera*/*Brettanomyces bruxellensis*, a spoilage wine yeast, on the surface of grape berries. *Microbiological Research*, **article in press**.

Romano, P. & Suzzi, G. (1993). Sulphur dioxide and wine microorganisms. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 373 - 393. New York: Taylor & Francis.

Romano, P., Fiore, C., Paraggio, M., Caruso, M. & Capece, A. (2003). Function of yeast species and strains in wine flavour. *International Journal of Food Microbiology*, **86**, 169-180.

Sato, H., Yanagida, F., Shinohara, T. & Yokotsuka, K. (2000). Restriction Fragment Length Polymorphism Analysis of 16s rRNA Genes in Lactic Acid Bacteria Isolated from Red Wine. *Journal of Bioscience and Bioengineering*, **90**, 335-337

Silla Santos, M.H. (1996). Biogenic amines: their importance in food. *International Journal of Food Microbiology*, **29**, 213-231.

Sponholz, W-R. (1993). Wine Spoilage by Microorganisms. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 395-420. New York: Taylor & Francis.

Ubeda, J.F. & Briones, A.I. (1999). Microbiological quality control of filtered and non-filtered wines. *Food Control*, **10**, 41-45.

Van Vuuren, H.J.J. & Dicks, L.M.T. (1993). *Leuconostoc oenos*: A review. *American Journal of Enology and Viticulture*, **44**, 99-112.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Friters, A., Pot, J., Paleman, J., Kuiper, M. & Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acid Research*, **23**, 4407-4414.

Xufre, A., Albergaria, H., Inácio, J., Spencer-Martins, I. & Gírio, F. (2006). Application of fluorescence in situ hybridisation (FISH) to the analysis of yeasts population dynamics in wines and laboratory grape must fermentations. *International Journal of Food Microbiology*, **108**, 376-384.

Zúñiga, M., Pardo, I. & Ferrer, S. (1993). An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. *International Journal of Food Microbiology*, **18**, 37-42.

CHAPTER 3

PCR-BASED DGGE FINGERPRINTING AND IDENTIFICATION OF THE MICROBIAL POPULATION IN SOUTH AFRICAN RED GRAPE MUST AND WINE

Abstract

Red wine production involves complex interactions between a variety of yeasts and bacteria. Conventional microbiological methods can be used to identify the different microorganisms present in wine, but prove to be time-consuming and certain microbial species may not grow on synthetic isolation media. The aim of this study was to evaluate the microbial population present in red grape must and wine by using polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE). Red wine of the Pinotage and Merlot variety was produced and samples taken throughout alcoholic and malo-lactic fermentation stages. DNA was extracted and a part of the small subunit ribosomal RNA (rRNA) was amplified using *Bacterial* and yeast specific primers. The PCR fragments were resolved by DGGE and unique fingerprints were obtained for the *Bacteria* and yeasts present in the Pinotage and Merlot wines. This method may serve as an alternative to conventional microbiological methods for the identification of the microbial species in red grape must and wine.

Introduction

The production of wine is a complex biochemical process involving interactions between a variety of different yeasts, bacteria and mycelial fungi (Fleet, 1993). The metabolism and interactions of these microbes influence the quality, aroma and flavour of the wine (Fleet, 2003). Winemaking involves two fermentations steps, the initial alcoholic fermentation, followed by the malo-lactic fermentation (MLF) (Boulton *et al*., 1996). During the alcoholic fermentation the yeasts convert the sugars in the grape must to ethanol and carbon dioxide (CO_2), as well as many secondary metabolic products that include higher alcohols, esters, organic acids and aldehydes

(Rapp & Versini, 1991; Fleet & Heard, 1993). The alcoholic fermentation can either take place by the inoculation of the grape must with strains of *Saccharomyces cerevisiae*, or spontaneously by the yeasts present on the grape surface and winery equipment (Fleet & Heard, 1993; Fugelsang, 1997). The non-*Saccharomyces* yeasts that initiate the alcoholic fermentation include species of the genera *Hanseniaspora*, *Candida*, *Metschnikowia*, *Hansenula*, *Zygosaccharomyces*, *Brettanomyces*, *Aureobasidium*, *Rhodotorula*, *Pichia*, *Kluyveromyces*, *Cryptococcus*, *Dekkera*, *Schizosaccharomyces*, *Torulaspora* and *Saccharomycodes* (Fleet & Heard, 1993; Fugelsang, 1997; Fleet, 2003; Querol *et al*., 2003; Romano *et al*., 2003).

Following on the alcoholic fermentation, MLF refers to the de-acidification of the wine by the conversion of L(-)-malic acid to L(+)-lactic acid by lactic acid bacteria (LAB). This second fermentation improves the aroma, flavour and microbial stability of the wine (Henick-Kling, 1993; Boulton *et al*., 1996). The LAB responsible for MLF in wine are members of the *Lactobacilliaceae*, characterised by the genus *Lactobacillus*, and the *Streptococcaceae*, characterised by the genera *Pediococcus* and *Leuconostoc* (Henick-Kling, 1993; Fugelsang, 1997). *Pediococcus damnosus*, *Leuconostoc mesenteroides* and *Oenococcus oeni* (previously *Leuconostoc oenos* (Lonvaud-Funel, 1999)) have been identified as the key LAB accountable for MLF (Lonvaud-Funel, 1999).

Conventional microbiological methods can be used to identify these different microorganisms present in wine, but prove to be time-consuming and often do not isolate all the microorganisms present due to the inability of some to grow on synthetic growth media (Heard & Fleet, 1986; Kopke *et al*., 2000). Molecular techniques offer new opportunities for identifying all the species present in a population (Ercolini, 2004). The aim of this study was to identify the microbial population present in South African red grape must and wine by using polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) fingerprinting and DNA sequencing. Red grape must and wine was also plated on selective growth media and the isolated microbes compared to the results from the PCR-based DGGE.

Material and methods

Red wine production and sampling

Pinotage wine was produced at the Department of Viticulture and Oenology, Stellenbosch University during the 2005 season. One part of the red grape must was inoculated with *S*. *cerevisiae* WE14 and the other left to spontaneously ferment. The fermentations were carried out at 25°C and daily samples of 50 ml were taken and frozen until the completion of the 6 day alcoholic fermentation. The inoculated alcoholic fermentation was also inoculated with *O*. *oeni* after the completion of the 6 d fermentation to commence MLF, while the spontaneous fermentation was left to undergo spontaneous MLF, both for a duration of 7 weeks. Weekly samples of 50 ml were taken and frozen until the completion of MLF.

Merlot wine was produced during the 2006 season at the experimental cellar, Department of Viticulture and Oenology, Stellenbosch University. The red grape must was chilled for 2 d at 15°C, after which it was inoculated with *S*. *cerevisiae* WE14. The alcoholic fermentation was carried out at 25°C and 50 ml samples were taken daily for the 9 d fermentation. After the completion of the alcoholic fermentation, the wine was left to undergo spontaneous MLF for 11 weeks and 50 ml samples were taken weekly.

DNA isolation

DNA was extracted from 2 ml of each sample of the Pinotage and Merlot must and wine. Prior to DNA isolation, the samples were filtered through a 0.22 µm filter (Lifesciences). DNA extractions were performed from the washed filter, as well as the filtrate.

DNA was isolated according to the modified method of Van Elsas *et al*. (1997). Two ml of the samples were centrifuged for 10 min at 5 900 x g after which the supernatant were discarded. The pellet, 0.6 g sterile glassbeads (0.2 – 0.3 mm in diameter) (Sigma), 800 µl phosphate buffer (1 part 120 mM NaH_2PO_4 (Merck) to 9 parts 120 mM Na_2HPO_4 (Merck); pH 8), 700 µl phenol (Fluka) and 100 µl 20% (m/v)

sodium dodecyl sulphate (SDS) (Merck) were vortexed for 2 min and incubated for 20 min at 60°C. This step was repeated twice. After incubation, the sample was centrifuged for 5 min at 1 500 x g. The aqueous phase was collected and the proteins were extracted with 600 µl phenol (Fluka). Further extraction was performed with a 600 µl phenol:chloroform:isoamylalcohol (25:24:1) mixture and repeated until the interphase was clean. The DNA was then precipitated with 0.1 volume 3 M sodium acetate (NaOAc) (pH 5.5) (Saarchem) and 0.6 volume isopropanol (Saarchem) on ice for 60 min. The mixture was centrifuged for 10 min at 15 000 x g, the pellet was washed with 70% (v/v) ethanol, and air-dried. The DNA was redissolved in 100 µl TE (10mM Tris (Fluka), 1mM EDTA (Merck); pH 8).

PCR-based DGGE analysis

The 5' end of the V3 variable region of the 16S ribosomal RNA (rRNA) gene was amplified using the *Bacteria* specific primers F341 (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') (GC clamp sequence is underlined) and R534 (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer *et al*., 1993). The PCR reactions were performed in a total volume of 25 µl containing 0.6 µM of each of the primers, 1.25 U *Taq* DNA polymerase (Southern Cross Biotechnologies), 1 x PCR reaction buffer containing $MgCl_2$ (Southern Cross Biotechnologies), 1 µl of 99% (v/v) dimethyl sulphoxide (DMSO) (Merck), 0.4 mM deoxyribonucleoside triphosphate (dNTPs) (Promega) and 1 µl of the extracted DNA. PCR reactions were performed in the Eppendorf Mastercycler Personal. An initial 4 min denaturation at 94°C was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 60 s and elongation at 72°C for 60 s and a final 5 min chain elongation at 72°C (Muyzer *et al*., 1993).

The 5'-end of the 26S rRNA gene was amplified using the yeast specific primers NL1 (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G-3' (GC clamp sequence is underlined) and LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (O'Donnell, 1993). The PCR reaction mixture was as previously described for the *Bacteria* except for using 1 x PCR reaction buffer without $MgCl_2$ (Southern Cross Biotechnologies), and the addition of 3

mM $MgCl_2$ (Southern Cross Biotechnologies). One µl of the extracted DNA from the Pinotage samples and 0.3 µl of the extracted DNA of the Merlot samples were used. The DNA was amplified during 30 cycles of denaturation at 95°C for 60 s, annealing at 52°C for 45 s and elongation at 72°C for 60 s. An initial 5 min denaturation at 95°C and a final 7 min chain elongation at 72°C were performed (Cocolin *et al*., 2000).

The PCR fragments were separated using DGGE, performed with the BioRad DCode Universal Mutation Detection System (Bio-Rad Laboratories). PCR samples were directly applied onto 8% (m/v) polyacrylamide gels in 1 x TAE buffer with a gradient of between 45 and 70% for both the *Bacterial* and yeast PCR fragments. The gradient was created by polyacrylamide, containing 1 to 100% denaturant (7 M urea and 40% (v/v) formamide). Electrophoresis was performed at a constant voltage of 130 mV for 5 h and a constant temperature of 60°C. The gel was stained with ethidium bromide and the fragments were visualized under UV light (Vilber Lourmat).

DNA sequencing

The dominant DGGE bands were punched from the gels and directly re-amplified using the primers R534 and F341 (without the GC-clamp) for the *Bacterial* fragments (Muyzer *et al*., 1993) and the primers LS2 and NL1 (without the GC-clamp) (O'Donnell, 1993) for the yeast fragments as previously described. All the PCR products were purified using the Sigma Spin Post-Reaction Purification Columns (Sigma Aldrich) as specified by the manufacturer. The PCR fragments were sequenced using the 3130XL Genetic Analyser (Applied Biosystems) at the DNA Sequencing Facility at Stellenbosch University. The sequences obtained were compared to sequences in the GenBank database using the BLASTn search option to verify the closest known relatives (Altschul *et al*., 1997).

Selective plating and identification of microbes in Merlot must and wine

A dilution series (10^{-1} to 10^{-9}) of the Merlot must and wine samples were done in sterile saline solution (SSS) (0.85% (m/v) NaCl (Merck)) and each dilution was spread plated (in duplicate) on four different growth media. Yeast-peptone-dextrose

(YPD)-agar (10 $g.L^{-1}$ yeast extract (Merck), 20 $g.L^{-1}$ peptone (Merck), 20 $g.L^{-1}$ dextrose (Merck) and 15 $g.L^{-1}$ bacteriological agar (Merck)), pH 6.5 (The South African Wine Laboratories Association, 2002) containing 30 $mg.L^{-1}$ chloramphenicol (Roche Diagnostics), was specific for yeasts. Glucose-yeast-calcium (GYC)-agar (50 $g.L^{-1}$ glucose (Merck), 10 $g.L^{-1}$ yeast extract (Merck), 30 $g.L^{-1}$ calcium carbonate ($CaCO_3$) (Merck) and 20 $g.L^{-1}$ bacteriological agar (Merck), pH 5.5 (Fugelsang, 1997; The South African Wine Laboratories Association, 2002), containing 30 $mg.L^{-1}$ chloramphenicol (Roche Diagnostics), was specific for the acetic acid bacteria (AAB). Wallerstein Laboratories Nutrient medium (WLN) (80 $g.L^{-1}$) (Merck), pH 5.8 (Fugelsang, 1997; The South African Wine Laboratories Association, 2002) was specific for yeasts and AAB. DeMan, Rogosa and Sharpe (MRS)-agar (50 $g.L^{-1}$ MRS broth (Merck) and 15 $g.L^{-1}$ bacteriological agar (Merck), pH 6.5 (The South African Wine Laboratories Association, 2002) containing 50 $mg.L^{-1}$ actistab (Gist-Brocades) was selective for lactic acid bacteria (LAB). The plates were incubated at 30°C for 5 d. Gram-staining was performed on single, pure colonies from the bacterial isolation medium.

The recovered cells from the plates were suspended in 30 µl ddH_2O and lysed for 5 min at 95°C followed by PCR amplification using the primers F8 (5' CAC GGA TCC AGA CTT TGA TYM TGG CTC AG -3') and R1512 (5'- GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT -3') (Felske *et al*., 1997). The PCR reactions were performed in a total reaction volume of 50 µl containing 0.4 µM of each of the primers, 2.5 U *Taq* DNA polymerase (Southern Cross Biotechnologies), 1 x PCR reaction buffer containing $MgCl_2$ (Southern Cross Biotechnologies), 0.4 mM dNTPs (Promega), 2 µl of 99% (v/v) DMSO (Merck) and 2 µl of the lysed cell mixture. The DNA was amplified during 35 cycles of denaturation at 92°C for 30 s, annealing at 54°C for 30 s and elongation at 68°C for 60 s. An initial 3 min denaturation at 92°C and a final 7 min chain elongation at 72°C were performed (Felske *et al*., 1997). The single PCR fragments were purified, sequenced and identified as previously described.

Results and discussion

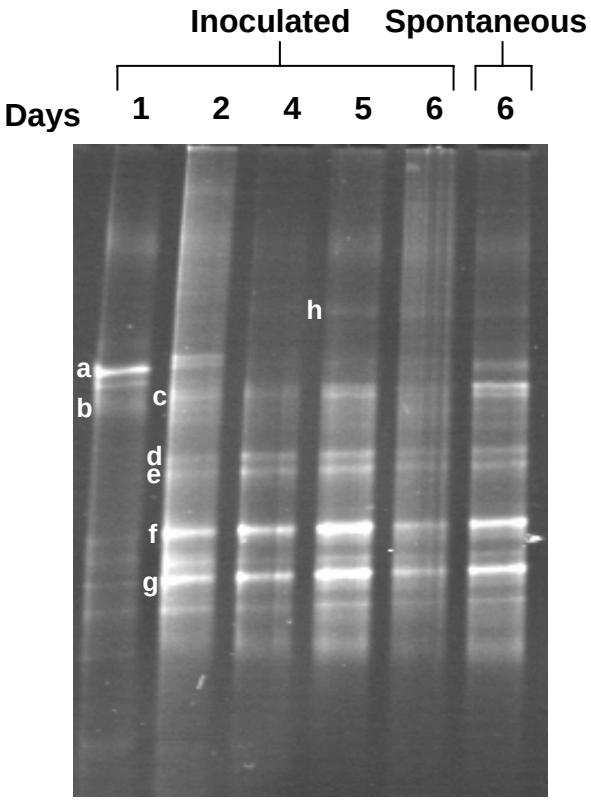
DGGE fingerprinting

Pinotage wine

Approximately 200 base pairs (bp) of the 5' end of the 16S rRNA gene were successfully amplified and resolved using DGGE. PCR-based DGGE analysis using *Bacteria* specific primers during alcoholic fermentation (Fig. 1) showed that the DGGE profile changed over the six day fermentation period and the DGGE fingerprint of day six of spontaneous alcoholic fermentation using *Bacteria* specific primers was similar to that of day six of the inoculated fermentation. The profile during spontaneous MLF (Fig. 2) was the same as for day six of inoculated and spontaneous alcoholic fermentation.

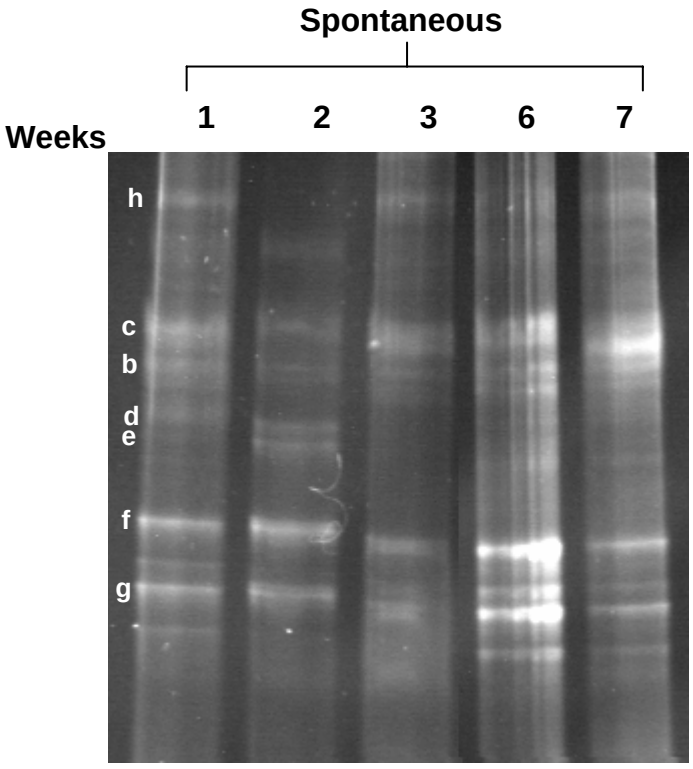
Band a disappeared after one day of inoculated alcoholic fermentation (Fig.1). This band was identified as *Lactobacillus plantarum* (100% homology, 142 out of 142 bases) (GenBank Accession number AY383631). Band b was present throughout the alcoholic fermentation (Fig.1) and MLF (Fig.2) processes and was identified as *Lactobacillus* sp. clone A12-10c (99% homology, 133 out of 134 bases) (GenBank Accession number DQ056428), closely related to *L*. *plantarum*. *Lactobacillus plantarum*, as well as other *Lactobacillus* species are commonly found on grapes, in the must and wine (Fugelsang, 1997). However, *L*. *plantarum* is not tolerant of high ethanol concentrations (Henick-Kling, 1993), which could provide a possible explanation for the decline of this microbe after one day of fermentation.

Bands c, d, e, f and g appeared on day two of alcoholic fermentation and were visible until the completion of MLF, while band h only appeared on day five of alcoholic fermentation and was present until the completion of MLF. Although these bands are not clearly visible due to the resolution of the photograph, visual inspection of the gel under the UV light showed these bands were present. These were punched from the gel to confirm results by sequencing. Band c (96% homology, 159 out of 165 bases) (GenBank Accession number DQ171118) was identified as an uncultured bacterial clone WS05A_D12 closely related to *Enterobacter sakazakii*.



a, b, c, d, e, f, g, h – DGGE bands identified using DNA sequencing

Figure 1 PCR-based DGGE analysis of South African Pinotage must and wine samples during alcoholic fermentation using *Bacteria* specific primers. Lanes 1 – 5: days one, two, four, five and six of the inoculated fermentation period. Lane 6: day six of the spontaneous fermentation.



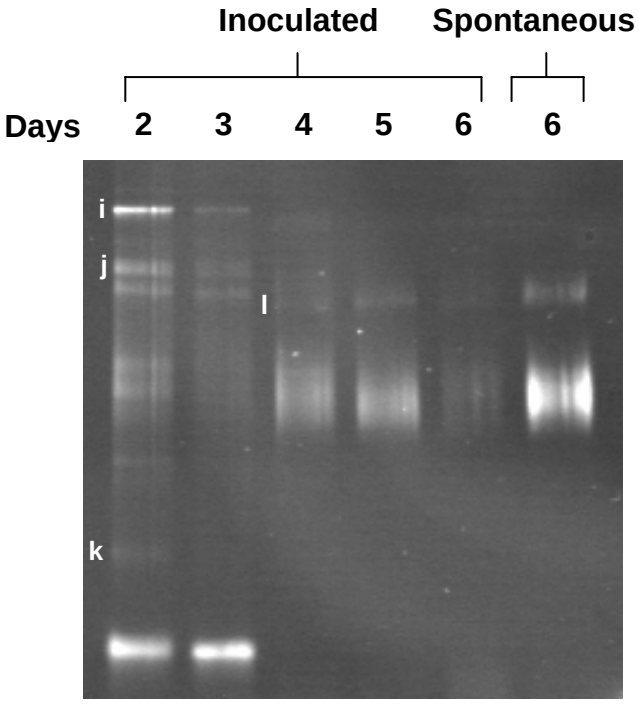
b, c, d, e, f, g, h – DGGE bands identified using DNA sequencing

Figure 2 PCR-based DGGE analysis of South African Pinotage must and wine samples during malo-lactic fermentation (MLF) using *Bacteria* specific primers. Lanes 1 – 5: weeks one, two, three, six and seven of spontaneous MLF.

Band e (100% homology, 165 out of 165 bases) (GenBank Accession number AB234526) was identified as an uncultured bacterial clone PBg1-024 closely related to *E. sakazakii*. Band g (99% homology, 142 out of 143 bases) (GenBank Accession number AY186083) was identified as an uncultured bacterial clone LB1B7 closely related to *Pantoea agglomerans* and band h (99% homology, 164 out of 165 bases) (GenBank Accession number AY376705) was identified as an uncultured bacterial clone O6 closely related to *E. sakazakii*. Band d was identified as *Enterobacter* sp. (100% homology, 165 out of 165 bases) (GenBank Accession number AY576743), closely related to *E. sakazakii* and band f was identified as *P. agglomerans* (100% homology, 151 out of 151 bases) (GenBank Accession number AY315454). These species are members of the family *Enterobacteriaceae* and are not commonly associated with wine. They are Gram-negative rods, facultative anaerobic and can grow over a wide temperature (25° - 37°C) and pH range (Holt *et al*., 1994; Krieg & Holt, 1984). The species of the family *Enterobacteriaceae* are ubiquitous in nature and have been isolated from soil, water, seeds, fruit and plant surfaces (Gavini *et al*., 1989). Therefore, the grapes could have come into contact with the soil during harvesting, during transport or from the winery environment. Since grapes are not washed prior to fermentation, these microbes could have entered the fermenting wine. In a study conducted by Venturini *et al*. (2002) they found several species of the *Enterobacteriaceae* family such as *P. agglomerans* and *Enterobacter cloacae* on cherries.

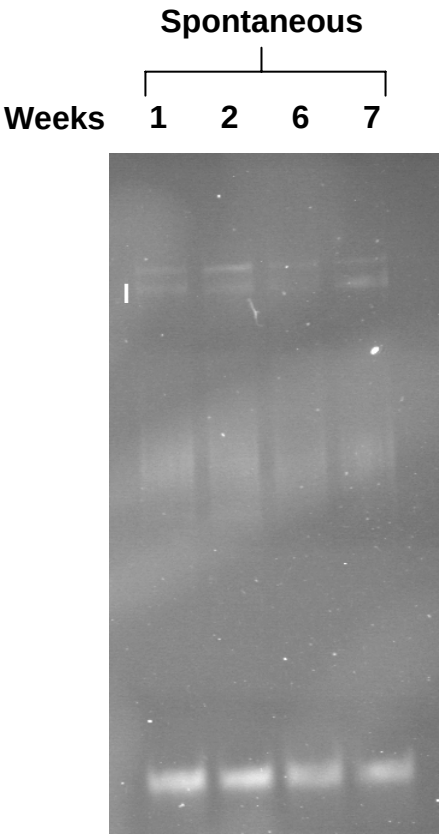
Approximately 250 bp of the 5’ end of the 26S rRNA gene were successfully amplified from the species present in the wine and resolved on a DGGE gel. PCR-based DGGE analysis using yeast specific primers during alcoholic fermentation (Fig. 3) showed that the profiles changed significantly during the course of the fermentation process. The DGGE fingerprint of day six of spontaneous alcoholic fermentation is similar to that of day six of the inoculated fermentation. The DGGE profile during spontaneous MLF (Fig. 4) showed fewer bands than the alcoholic fermentation profile.

Band i was identified as *Hanseniaspora uvarum* (99% homology, 161 out of 163 bases) (GenBank Accession number HUU84229) and band j as



i, j, k, l – DGGE bands identified using DNA sequencing

Figure 3 PCR-based DGGE analysis of South African Pinotage must and wine samples during alcoholic fermentation using yeast specific primers. Lanes 1 – 5: days two to six of inoculated alcoholic fermentation, Lane 6: day six of spontaneous alcoholic fermentation.



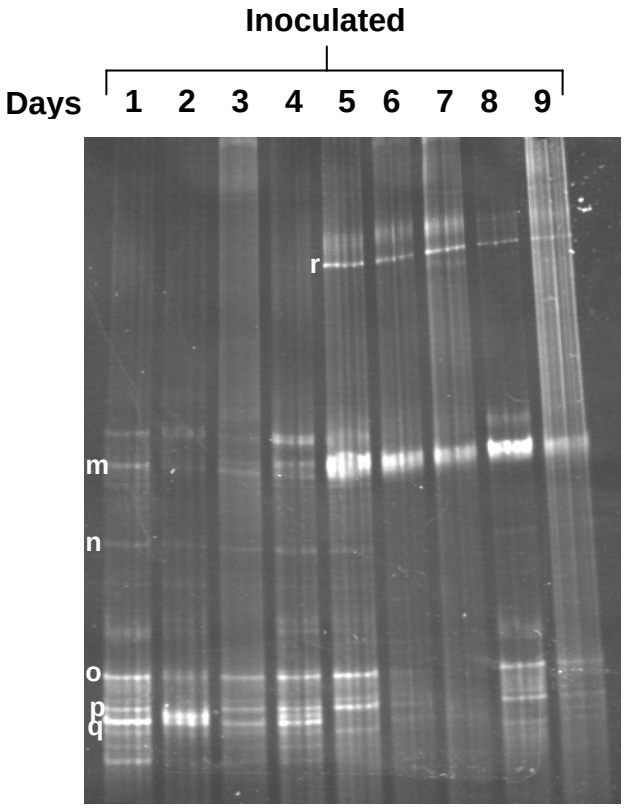
I – DGGE band identified using DNA sequencing

Figure 4 PCR-based DGGE analysis of South African Pinotage must and wine samples during malo-lactic fermentation (MLF) using yeast specific primers. Lanes 1 – 4: weeks one, two, six and seven of spontaneous MLF.

Zygosaccharomyces rouxii (95% homology, 140 out of 148 bases) (GenBank Accession number AJ966531). These two microbes died-off after three days of alcoholic fermentation (Fig.3). Band k was identified as *Issatchenkia orientalis* (100% homology, 180 out of 180 bases) (GenBank Accession number AY601160) which died-off after two days of alcoholic fermentation (Fig.3). This microbe is the teleomorph of *Candida krusei* which has previously been isolated from wine (Abranches *et al*., 1998; Clemente-Jimenez *et al*., 2004). Band l was identified as *Saccharomyces cerevisiae* (100% homology, 186 out of 186 bases) (GenBank Accession number AY130346). *Hanseniaspora uvarum*, *Z*. *rouxii* and *I*. *orientalis* (bands i, j and k) are well-known non-*Saccharomyces* yeasts and are normally found to be present at the beginning stages of the alcoholic fermentation process (Fleet & Heard, 1993; Fugelsang, 1997; Fleet, 2003). *Saccharomyces cerevisiae* (band l) only appeared after four days of alcoholic fermentation (Fig.3) and was present up to the completion of MLF (Fig.4). It is typical of wine fermentation that the non-*Saccharomyces* yeasts initiate the alcoholic fermentation process, but die-off within the first two to three days of fermentation, after which *S. cerevisiae* completes the fermentation. The non-*Saccharomyces* yeast species are not tolerant of ethanol concentrations higher than *ca* 5 - 7% (v/v). It is the ethanol produced by *S*. *cerevisiae*, together with the fermentation temperature, which controls the growth of the non-*Saccharomyces* yeasts (Fleet & Heard, 1993; Fleet, 2003).

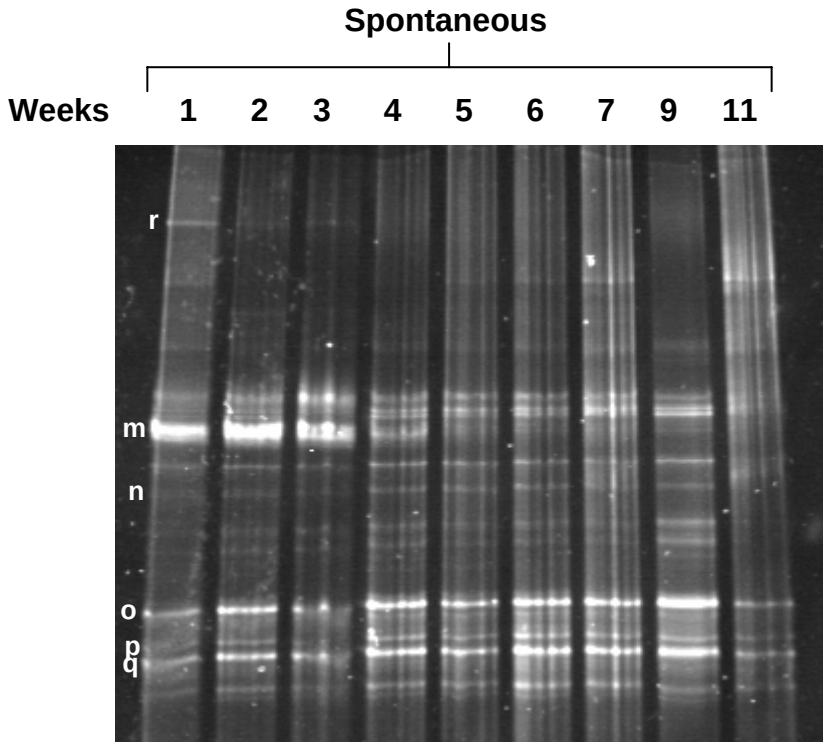
Merlot wine

PCR-based DGGE analysis using *Bacteria* specific primers during inoculated alcoholic fermentation (Fig. 5) showed that the DGGE profile was more or less the same for the nine day fermentation period, except for one band that appeared from day five. The profile during spontaneous MLF (Fig. 6) was the same for day nine of the alcoholic fermentation, except for one band that disappeared during the ninth week of the MLF. Although this is not clearly visible due to the resolution of the photograph, visual inspection of the gel under the UV light showed these bands were present. These were punched from the gel to confirm results by sequencing. Band m was present from the start of the alcoholic fermentation and died-off during week five



m, n, o, p, q, r – DGGE bands identified using DNA sequencing

Figure 5 PCR-based DGGE analysis of South African Merlot must and wine samples during alcoholic fermentation using *Bacteria* specific primers. Lanes 1 – 9: days one to nine of inoculated alcoholic fermentation.



m, n, o, p, q, r – DGGE bands identified using DNA sequencing

Figure 6 PCR-based DGGE analysis of South African Merlot must and wine samples during malo-lactic fermentation (MLF) using *Bacteria* specific primers. Lanes 1 – 9: weeks one to seven, nine and eleven of spontaneous MLF.

of MLF. This band was presumptively identified as an uncultured bacterium clone DBF1G4 (98% homology, 50 out of 51 bases) (GenBank Accession number DQ190157).

Band n was present throughout the alcoholic fermentation process (Fig.5). Although it seems like this band disappeared on day six and seven, this is only due to the resolution of the photograph. Visual inspection showed that they were present and was confirmed by punching these bands from the gel followed by sequencing. Band n only disappeared during week nine of MLF (Fig.6). This band was identified as *E. sakazakii* (96% homology, 148 out of 154 bases) (GenBank Accession number AY752939). Bands o, p and q was present throughout both fermentation processes. Band p was identified as *E. sakazakii* (97% homology, 147 out of 151 bases) (GenBank Accession number AY752939) and band o was identified as *P. agglomerans* (98% homology, 162 out of 165 bases) (GenBank Accession number DQ530141). Band q was identified as an uncultured bacterium clone RSA1 (95% homology, 105 out of 111 bases) (GenBank Accession number DQ009673) closely related to *P. agglomerans*.

Band r only appeared on day five of alcoholic fermentation and died off during the third week of MLF. This band was identified as an uncultured bacterium clone RSA1 (95% homology, 166 out of 174 bases) (GenBank Accession number DQ009673) closely related to *P. agglomerans*. The presence of *Enterobacteriaceae* shows that contamination could have come from the soil or plant leaves (Gavini *et al*., 1989). Interspecies heterogeneity of the 16S rRNA gene sequence could lead to the detection of several bands when only one species is present (Coenye & Vandamme, 2003), which can explain why there are two bands representing *E. sakazakii* and two bands representing uncultured bacterium clone RSA1 present in the DGGE profile.

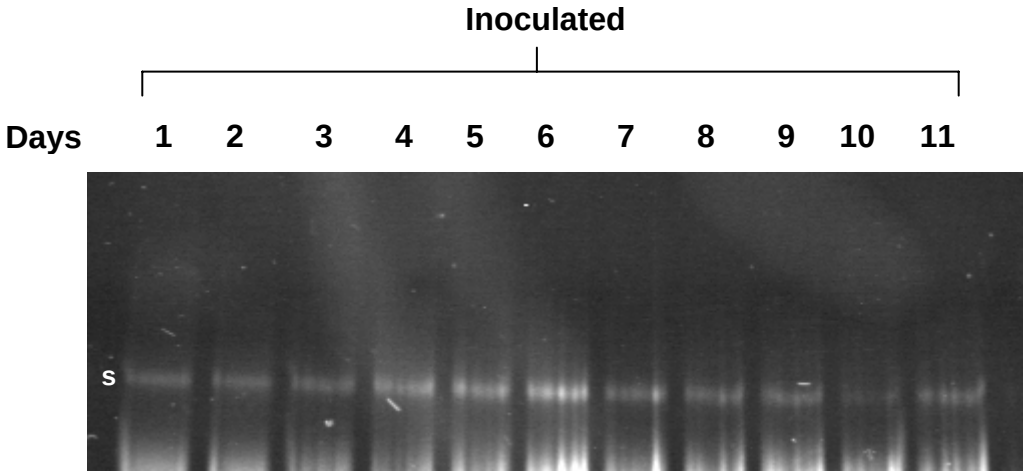
By comparison of the results from the PCR-based DGGE technique and the conventional plating method of the Merlot wine samples on the different media, it was shown that only one bacterial species could be identified from the MRS media. By Gram-staining this microbe was recognised as a Gram-positive rod and following sequencing it was identified as *Lactobacillus brevis* (100% homology, 155 out of 155 bases) (GenBank Accession number DQ523492). The fact that this microbe could

not be identified using the PCR-based DGGE technique, could be due to the low concentration of the microbe in the must and wine samples (Ercolini, 2004, Savazzini & Martinelli, 2005).

PCR-based DGGE analysis using yeast specific primers during alcoholic fermentation (Fig. 7) and MLF (Fig. 8) showed that only one band was present during both fermentation processes and that this microbe died-off during week eleven of MLF. Band s was identified as *S. cerevisiae* (100% homology, 186 out of 186 bases) (GenBank Accession number AY601161). By comparison of the results from the PCR-based DGGE technique and that of the conventional cultivation and plating onto the different media, the results of the DGGE technique were confirmed. *Saccharomyces cerevisiae* (100% homology, 157 out of 157 bases) (GenBank Accession number AY601161) was isolated from the WLN and YPD media and identified by sequencing.

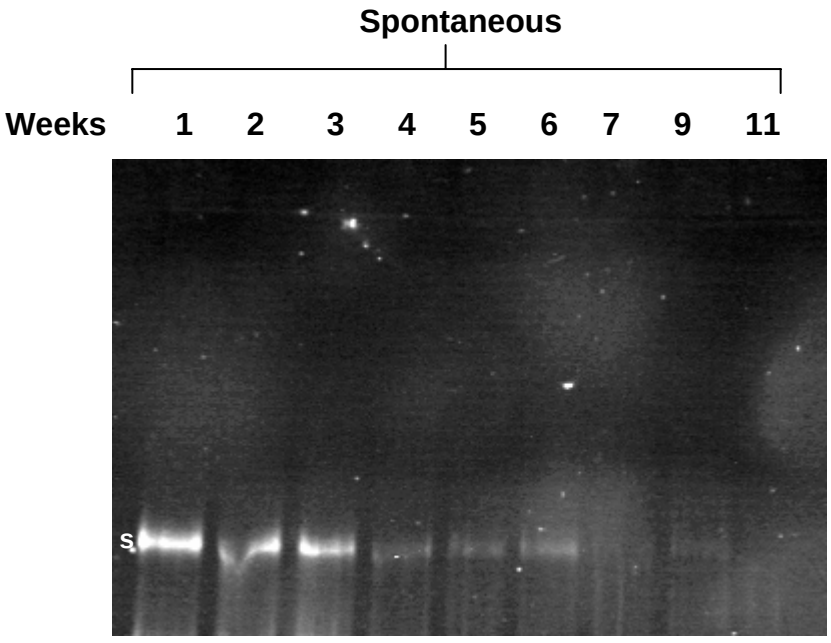
Conclusions

Analysis of both Pinotage and Merlot wines during their alcoholic fermentation and MLF showed that the fermentations are carried out by complex microbial populations which consist of a succession of yeast and bacterial species. *Saccharomyces cerevisiae* was shown to be the principle wine yeast by PCR-DGGE and was also isolated from selective plating, but there are also many other microorganisms that may have contributed to the wine flavour profile and quality (Fleet & Heard, 1993). Analysis of the wine samples also showed many uncultured microbes and microbes from the *Enterobacteriaceae* family to be present in the wine. These microbes could have contaminated the wine at the time of harvesting the grapes or from the winery environment. The comparison of the Merlot must and wine samples using the PCR-based DGGE technique and selective plating showed that none of these microbes identified using the DGGE technique could be isolated using conventional plating. However, *L. brevis* was isolated from selective plating and not by using PCR-DGGE. From these results, PCR-based DGGE showed to be a possible alternative to conventional microbiological methods for the identification of the microbial species in red grape must and wine during the two fermentation processes.



s – DGGE band identified using DNA sequencing

Figure 7 PCR-based DGGE analysis of South African Merlot must and wine samples during alcoholic fermentation using yeast specific primers. Lanes 1 – 11: days one to eleven of inoculated alcoholic fermentation.



s – DGGE band identified using DNA sequencing

Figure 8 PCR-based DGGE analysis of South African Merlot must and wine samples during malo-lactic fermentation (MLF) using yeast specific primers. Lanes 1 – 9: weeks one to seven, nine and eleven of spontaneous MLF.

References

Abranches, J., Valente, P., Nóbrega, H.N., Fernandez, F.A.S., Mendonça-Hagler, L.C. & Hagler, A.N. (1998). Yeast diversity and killer activity dispersed in fecal pellets from marsupials and rodents in a Brazilian tropical habitat mosaic. *FEMS Microbiology Ecology*, **26**, 27-33.

Altschul, S.F., Madden, T.L. Schaffer, A.A., Zhang, Z., Miller, W. & Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acid Research*, **25**, 3389-3402.

Beer, S.V., Rundle, J.R. & Norelli, J.L. (1984). Recent progress in the development of biological control for fire blight – a review. *Acta Horticulturae*. **151**,195-201.

Boulton, R.B., Singleton, V.L., Bisson, L.F. & Kunkee, R.E. (1996). Principles and practices of winemaking. Pp. 132-133, 245, 247. New York: Chapman & Hall.

Clemente-Jimenez, J.M., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Las Heras-Vázquez, F.J. & Rodríguez-Vico, F. (2004). Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. *Food Microbiology*, **21**, 149-155.

Cocolin, L., Bisson, L.F. & Mills, D.A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiology Letters*, **189**, 81-87.

Coenye, T. & Vandamme, P. (2003). Intragenomic heterogeneity between multiple 16S ribosomal RNA operon in sequenced bacterial genomes. *FEMS Microbiology Letters*, **228**, 45-48.

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, **56**, 297-314.

Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E. & Akkermans, A.D.L. (1997). Ribosome analysis reveals prominent activity of an uncultured member of the class *Acinetobacteria* in grassland soils. *Microbiology*, **143**, 2983-2989.

Fleet, G.H. (1993). The microorganisms of winemaking – isolation, enumeration and identification. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 1-26. New York: Taylor & Francis.

Fleet, G.H. (2003). Yeast interactions and wine flavour. *International Journal of Food Microbiology*, **86**, 11-22.

Fleet, G.H. & Heard, G.M. (1993). Yeasts-growth during fermentation. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 27-54. New York: Taylor & Francis.

Fugelsang, K.C. (1997). Wine Microbiology. Pp. 3-47, 48-67, 68-111, 132-137. New York: Chapman & Hall.

Gavini, F., Mergaert, J., Beiji, A., Mielcarek, C., Izard, D., Kersters, K. & De Ley, J. (1989). Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea agglomerans* comb. nov. and Description of *Pantoea dispersa* sp. nov. *International Journal of Systematic Bacteriology*, 39, 337-345.

Heard, G.M. & Fleet, G.H. (1986). Evaluation of selective media for enumeration of yeasts during wine fermentation. *Journal of Applied Bacteriology*, **60**, 477-481.

Henick-Kling, T. (1993). Malo-lactic Fermentation. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 289-326. New York: Taylor & Francis.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. & Williams, S.T. (1994). Bergey's Manual of Determinative bacteriology, 9th ed. Pp. 175, 178, 184. Maryland: Williams & Wilkens.

Jay, J.M. (1998). Modern Food Microbiology, 5th ed. Pp. 390-392. Maryland: Aspen Publishers Inc.

Johnson, K.B., Stockwell, V.O., McLaughlin, R.J., Sugar, D., Loper, J.E. & Roberts, R.G. (1993). Effect of antagonistic bacteria on establishment of honey bee-dispersed *Erwinia amylovora* in pear blossoms and on fire blight control. *Phytopathology*, **83**, 995-1002.

Kopke, C., Cristovão, A., Prata, A.M., Silva Pereira, C., Figueiredo Marques, J.J. & San Romão, M.V. (2000). Microbiological control of wine. The application of epfiluorescence microscopy method as a rapid technique. *Food Microbiology*, **17**, 257-260.

Krieg, N.R. & Holt, J.G. (1984). Bergey's Manual of Systematic bacteriology, Vol. 1. Pp. 408 - 409. London: Williams & Wilkens.

Lonvaud-Funel, A. (1999). Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoek*, **76**, 317-331.

Muyzer, G., de Waal, E.C. & Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase

chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, **59**, 695-700.

O'Donnell, K. (1993). Fusarium and its near relatives, In: *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics* (edited by Reynolds, D.R. & Taylor, J.W.). Pp. 225-233. Wallingford, UK: CAB International.

Querol, A., Fernández-Espinar, M.T., Ií del Olmo, M. & Barrio, E. (2003). Adaptive evolution of wine yeast. *International Journal of Food Microbiology*, **86**, 3-10.

Rapp, A. & Versini, G. (1991). Influence of nitrogen compounds in grapes on aroma compounds in wine. In: *Proceedings of the International symposium on nitrogen in grapes and wines* (edited by RANTZ). Pp. 156-164. Davis, CA: American Society for Enology and Viticulture.

Romano, P., Fiore, C., Paraggio, M., Caruso, M. & Capece, A. (2003). Function of yeast species and strains in wine flavour. *International Journal of Food Microbiology*, **86**, 169-180.

Savazzini, F. & Martinelli, L. (2006). DNA analysis in wines: Development of methods for enhanced extraction and real-time polymerase chain reaction quantification. *Analytica Chimica Acta*, **563**, 274-282.

The South African Wine Laboratories Association. (2002). *Methods of analysis for wine laboratories*. South Africa: South African Society for Enology and Viticulture.

Van Elsas, J.D., Matynen, V. & Wolters, A. (1997). Soil DNA extraction and assessment of the fate of *Mycobacterium chlorophenolicum* strain PCR-1 in different soils by 16S ribosomal RNA gene sequence based most-probable-number PCR immunofluorensence. *Biological Fertilization*, **24**, 188-195.

Venturini, M.E., Oria, R. & Blanco, D. (2002). Microflora of two varieties of sweet cherries: Burlat and Sweetheart. *Food Microbiology*, **19**, 15-21.

CHAPTER 4

PCR-BASED DGGE FINGERPRINTING AND IDENTIFICATION OF THE MICROBES PRESENT IN SPOILT COMMERCIAL SOUTH AFRICAN WINE

Abstract

Yeasts and bacteria associated with wine can produce undesirable metabolic by-products which, when present at high concentrations, can result in spoilage of the wine. Although conventional microbiological methods are currently used for the identification of these spoilage microbes, these prove to be time-consuming and certain microbes do not grow on synthetic media. The aim of this study was to identify the microbes present in spoilt commercial wines using a non-culturing approach, polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE), and to compare these results with conventional microbiological plating. Four wine samples (RW1, RW2, RoW1 and WW1) were found to be spoilt by *Saccharomyces cerevisiae*, while a fifth wine sample (RW3) was found to be spoilt by an *Acetobacter* sp. using PCR-based DGGE. From all five samples, members of the family *Enterobacteriaceae* were identified using PCR-based DGGE, while *Enterobacter sakazakii* was isolated from RW1 using conventional plating. The members of the family *Enterobacteriaceae* could possibly have contributed to the spoilage of the wine by producing undesirable secondary metabolites. From these results PCR-based DGGE proved to be a possible alternative to conventional microbiological methods for the detection of spoilage microbes in wine.

Introduction

Wine spoilage can occur during three different stages of the wine making process. The first stage is microbiological contamination of the grapes by certain yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and mycelial fungi. Secondly, spoilage can occur due to microbial contamination during fermentation, and thirdly, spoilage can

occur in the bottled wine, where the wine can act as a growth substrate for undesirable yeasts or bacteria. The uncontrolled growth of microbes during any of the three stages results in the production of undesirable metabolic by-products which can affect the wine quality, aroma and appearance (Sponholz, 1993).

Even though species of the yeast genus *Brettanomyces* represent some of the most important spoilage yeasts associated with wine (Sponholz, 1993), there are many other yeast species associated with wine spoilage. Winemakers add specialised strains of *Saccharomyces cerevisiae* to the grape must to encourage the alcoholic fermentation and to improve control over the process (Fugelsang, 1997; Querol *et al*., 2003), but the presence of this yeast is considered a spoilage factor when present in bottled wine (Fleet, 2003). Spoilage caused by *S. cerevisiae* is due to the re-fermentation of the residual sugars in wines (Loureiro & Malfeito-Ferreira, 2003). *Saccharomyces cerevisiae* can also cause spoilage due to the production of different levels of desirable or undesirable secondary metabolites, such as varying levels of higher alcohols, acetaldehyde and acetic acid (Romano *et al*., 2003).

Apart from yeasts, bacteria can also cause spoilage of bottled wine. Although LAB play an important role in improving the organoleptic quality of the wine, these microbes can cause spoilage by the production of D-lactic acid, acetic acid and diacetyl (Sponholz, 1993). Certain AAB spoil the wine by metabolising ethanol to acetic acid and acetaldehyde (Drysdale & Fleet, 1988; Fugelsang, 1997).

Although conventional microbiological methods can be used to identify the different microbes present in wine, these prove to be time-consuming and certain microbial species can not be isolated from synthetic growth media (Heard & Fleet, 1986; Kopke *et al*., 2000). Molecular techniques have proven to be reliable and rapid alternatives for conventional microbiological plating (Ercolini, 2004). Therefore, the aim of this study was to identify the microbes present in spoilt commercial South African wines by using polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE). The spoilt wine samples were also plated on selective growth media and the isolated microbes compared to the results from the PCR-based DGGE.

Material and methods

DNA isolation

Five spoilt commercial wine samples of unknown grape varieties were obtained from the Department of Viticulture and Oenology, Stellenbosch University. These wines were spoilt based on sensory characteristics and included three spoilt red wines (RW1, RW2 and RW3), one spoilt white wine (WW1) and one spoilt rosé wine (RoW1). Prior to DNA isolation, the samples were filtered through a 0.22 µm filter (Lifesciences). DNA extractions were made from the washed filter, as well as from the filtrate.

DNA was isolated according to the modified method of Van Elsas *et al*. (1997). Two ml of the spoilt wines were centrifuged for 10 min at 5 900 x g after which the supernatant was discarded. The pellet, 0.6 g sterile glass beads (0.2 – 0.3 mm in diameter) (Sigma), 800 µl phosphate buffer (1 part 120 mM NaH_2PO_4 (Merck) to 9 parts 120 mM Na_2HPO_4 (Merck); pH 8), 700 µl phenol (Fluka) and 100 µl 20% (m/v) sodium dodecyl sulphate (SDS) (Merck) were vortexed for 2 min and incubated for 20 min at 60^{o}C. This step was repeated twice. After incubation, the sample was centrifuged for 5 min at 1 500 x g. The aqueous phase was collected and the proteins were extracted with 600 µl phenol (Fluka). Further extraction was performed with a 600 µl phenol:chloroform:isoamylalcohol (25:24:1) mixture and repeated until the interphase was clean. The DNA was then precipitated with 0.1 volume 3 M sodium acetate (NaOAc) (pH 5.5) (Saarchem) and 0.6 volume isopropanol (Saarchem) on ice for 60 min. The mixture was centrifuged for 10 min at 15 000 x g, the pellet was washed with 70% (v/v) ethanol, and air-dried. The DNA was redissolved in 100 µl TE (10mM Tris (Fluka), 1mM EDTA (Merck); pH 8).

PCR-based DGGE analysis

The 5’ end of the V3 variable region of the 16S ribosomal RNA (rRNA) gene was amplified using the *Bacteria* specific primers F341 (5’-CGC CCG CCG CGC GCG GCG

GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') (GC clamp sequence is underlined) and R534 (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer *et al*., 1993). The PCR reactions were performed in a total volume of 25 µl containing 0.6 µM of each of the primers, 1.25 U *Taq* DNA polymerase (Southern Cross Biotechnologies), 1 x PCR reaction buffer containing $MgCl_2$ (Southern Cross Biotechnologies), 1 µl of 99% (v/v) dimethyl sulphoxide (DMSO) (Merck), 0.4 mM deoxyribonucleoside triphosphate (dNTPs) (Promega) and 1 µl of the extracted DNA. PCR reactions were performed in the Eppendorf Mastercycler Personal. An initial 4 min denaturation at 94°C was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 60 s and elongation at 72°C for 60 s and a final 5 min chain elongation at 72°C (Muyzer *et al*., 1993).

The 5'-end of the 26S rRNA gene was amplified using the yeast specific primers NL1 (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G-3' (GC clamp sequence is underlined) and LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (O'Donnell, 1993). The PCR reaction mixture was as previously described for the *Bacterial* amplification except for using 1 x PCR reaction buffer without $MgCl_2$ (Southern Cross Biotechnologies) and the addition of 3 mM $MgCl_2$ (Southern Cross Biotechnologies). The DNA was amplified during 30 cycles of denaturation at 95°C for 60 s, annealing at 52°C for 45 s and elongation at 72°C for 60 s. An initial 5 min denaturation at 95°C and a final 7 min chain elongation at 72°C were performed (Cocolin *et al*., 2000).

The PCR fragments were separated using DGGE, performed with the BioRad DCode Universal Mutation Detection System (Bio-Rad Laboratories). PCR samples were directly applied onto 8% (m/v) polyacrylamide gels in 1 x TAE buffer with a gradient of between 45 and 70% for both the *Bacterial* and yeast PCR fragments. The gradient was created by polyacrylamide, containing 1 to 100% denaturant (7 M urea and 40% (v/v) formamide). Electrophoresis was performed at a constant voltage of 130 mV for 5 h and a constant temperature of 60°C. The gel was stained with ethidium bromide and the fragments were visualized under UV light (Vilber Lourmat).

DNA sequencing

The DGGE bands were punched from the gels and directly re-amplified using the primers F341 (without the GC-clamp) and R534 for the *Bacterial* fragments (Muyzer *et al*., 1993) and the primers NL1 (without the GC-clamp) and LS2 (O'Donnell, 1993) for the yeast fragments as previously described. All the PCR products were purified using the Sigma Spin Post-Reaction Purification Columns (Sigma) as specified by the manufacturer. The PCR fragments were sequenced using the 3130XL Genetic Analyser (Applied Biosystems) at the DNA Sequencing Facility, Stellenbosch University. The sequences obtained were compared to sequences in GenBank using the BLASTn search option to verify the closest known relatives (Altschul *et al*., 1997).

Selective platings and identification of spoilage microbes

A dilution series (10^{-1} to 10^{-9}) of the red (RW2 and RW3) and white (WW1) wines were done in sterile saline solution (SSS) (0.85% (m/v) NaCl (Merck)) and each dilution was spread plated (in duplicate) on four different growth media. Yeast-peptone-dextrose (YPD)-agar (10 $g.L^{-1}$ yeast extract (Merck), 20 $g.L^{-1}$ peptone (Merck), 20 $g.L^{-1}$ dextrose (Merck) and 15 $g.L^{-1}$ bacteriological agar (Merck)), pH 6.5 (The South African Wine Laboratories Association, 2002) containing 30 $mg.L^{-1}$ chloramphenicol (Roche Diagnostics), was specific for yeasts. Glucose-yeast-calcium (GYC)-agar (50 $g.L^{-1}$ glucose (Merck), 10 $g.L^{-1}$ yeast extract (Merck), 30 $g.L^{-1}$ calcium carbonate ($CaCO_3$) (Merck) and 20 $g.L^{-1}$ bacteriological agar (Merck)), pH 5.5 (Fugelsang, 1997; The South African Wine Laboratories Association, 2002) containing 30 $mg.L^{-1}$ chloramphenicol (Roche Diagnostics), was specific for the AAB. Wallerstein Laboratories Nutrient (WLN) medium (80 $g.L^{-1}$) (Merck), pH 5.8 (Fugelsang, 1997; The South African Wine Laboratories Association, 2002) was specific for yeasts and AAB. DeMan, Rogosa and Sharpe (MRS) agar (50 $g.L^{-1}$ MRS broth (Merck) and 15 $g.L^{-1}$ bacteriological agar (Merck)), pH 6.5 (The South African Wine Laboratories Association, 2002) containing 50

$mg.L^{-1}$ actistab (Gist-Brocades), was selective for LAB. The plates were incubated at 30°C for 5 d. Gram-staining was performed on single, pure colonies.

The recovered cells from the plates were suspended in 30 µl ddH_2O and lysed for 5 min at 95°C, followed by PCR amplification using the primers F8 (5' CAC GGA TCC AGA CTT TGA TYM TGG CTC AG -3') and R1512 (5'- GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT -3') (Felske *et al*., 1997). The PCR reactions were performed in a total reaction volume of 50 µl containing 0.4 µM of each of the primers, 2.5 U *Taq* DNA polymerase (Southern Cross Biotechnologies), 1 x PCR reaction buffer containing $MgCl_2$ (Southern Cross Biotechnologies), 0.4 mM dNTPs (Promega), 2 µl of 99% (v/v) DMSO (Merck) and 2 µl of the lysed cell mixture. The DNA was amplified during 35 cycles of denaturation at 92°C for 30 s, annealing at 54°C for 30 s and elongation at 68°C for 60 s. An initial 3 min denaturation at 92°C and a final 7 min chain elongation at 72°C were performed (Felske *et al*., 1997). The single PCR fragments were sequenced and identified as previously described.

In order to confirm the presence of *Enterobacter sakazakii*, wine sample RW1 was filtrated through a 0.22 µm filter (Lifesciences) and the filter was transferred to 90 ml *Enterobacteriaceae* enrichment (EE) broth (Oxoid) and incubated overnight at 37°C. After incubation, 0.1 ml of the enrichment culture was spread-plated onto Tryptic Soy Agar (TSA) (Oxoid) and incubated at 25°C for 72 h. Yellow colonies were streaked until pure colonies were obtained, after which a Gram-stain was performed and the isolates identified using the API 20E system (API System S.A. La Balme le Grottes, 38390, Montalieu, France).

An *E. sakazakii* detection PCR reaction using the primers Esak2 (5' CCC GCA TCT CTG CAG GAT TCT C 3') and Esak3 (5' CTA ATA CCG CAT AAC GTC TAC G 3') (Keyser *et al*., 2003) was performed in a total reaction volume of 25 µl containing 0.2 µM of each of the primers, 1 U *Taq* DNA polymerase (Southern Cross Biotechnologies), 1 x PCR reaction buffer without $MgCl_2$ (Southern Cross Biotechnologies), 1.5 mM of $MgCl_2$ (Southern Cross Biotechnologies), 1 µl of 99% (v/v) DMSO (Merck), 0.4 mM dNTPs (Promega) and 1 µl of the extracted DNA. A pure culture of *E. sakazakii* (1039, University of Stellenbosch Food Science Culture Collection) was used as a positive

control. An initial 2 min denaturation at 95°C was followed by 35 cycles of denaturation at 95°C for 35 s, annealing at 61°C for 60 s and elongation at 72°C for 60 s and a final 10 min chain elongation at 72°C. The PCR products were separated on a 1% (m/v) agarose gel and visualised under UV light (Vilber Lourmat).

Results and discussion

DGGE fingerprinting and conventional platings

Approximately 200 base pairs (bp) of the 5' end of the 16S rRNA gene and approximately 250 bp of the 5' end of the 26S rRNA gene were successfully amplified from all five wine samples. The PCR products were then successfully resolved using DGGE.

RW1

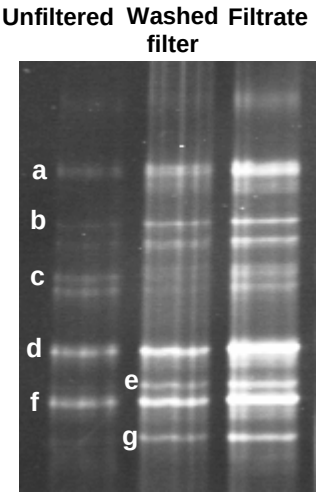
PCR-based DGGE analysis using *Bacteria* specific primers (Fig. 1) shows the profiles of the unfiltered wine sample, the washed filter of the filtrated wine sample and the filtrate. It is clear from these profiles that by filtrating the wine prior to DNA extraction, the bands are more visible possibly due to a higher concentration of the microbial cells or by eliminating some of the compounds that may inhibit DNA extraction. From these profiles seven bands could be identified.

Band a was identified as a gamma proteobacterium Y-134 (95% homology, 145 out of 152 bases) (GenBank Accession number AB096215), closely related to *Trabulsiella guamensis*. This microbe is a member of the *Enterobacteriaceae* and has previously been isolated from soil and dust (McWhorter *et al*., 1991). The *Enterobacteriaceae* are Gram-negative rods, facultative anaerobic and can grow over a wide temperature range (25° - 37°C) (Holt *et al*., 1994; Krieg & Holt, 1984). The species of the family *Enterobacteriaceae* are ubiquitous in nature and have been isolated from soil, water, seeds, fruit and plant surfaces (Gavini *et al*., 1989). The grapes used in wine-making could have come into contact with the soil during harvesting or during

transport. Since grapes are not washed prior to fermentation, these microbes could have entered the fermenting wine and survived in the bottled wine.

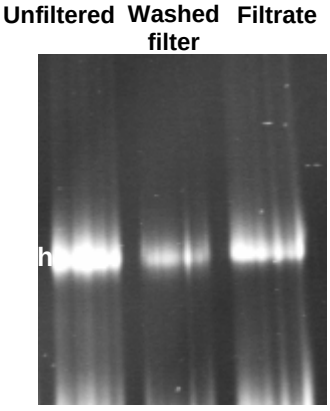
Band b was identified as an uncultured bacterial clone PBg1-024 (100% homology, 153 out of 153 bases) (GenBank Accession number AY91163), closely related to *Enterobacter sakazakii*. Band c was identified as *Pantoea agglomerans* (99% homology, 151 out of 152 bases) (GenBank Accession number AY691545), as well as band d (99% homology, 121 out of 122 bases) (GenBank Accession number AY691545). *Pantoea agglomerans* is a member of the *Enterobacteriaceae* and could have entered the wine by contact of the grapes with the soil or by the irrigation water (Gavini *et al*., 1989). Band e was identified as *E. sakazakii* (98% homology, 149 out of 151 bases) (GenBank Accession number AY752940). Band f (100% homology, 154 out of 154 bases) (GenBank Accession number AY791163) and band g (99% homology, 152 out of 153 bases) (GenBank Accession number AY791163) were both identified as an uncultured bacterial clone PBg1-024 closely related to *E. sakazakii*. The detection of several bands when only one species is present can be explained by interspecies heterogeneity of the 16S rRNA gene sequence (Coenye & Vandamme, 2003), which can explain why three bands representing uncultured bacterial clone PBg1-024 and two bands representing *P. agglomerans* are present in the DGGE profile.

The presence of *E. sakazakii* in wine has not previously been reported, therefore, confirmation of the presence of this microbe in the red wine sample (RW1) was done when it was plated onto TSA plates and the yellow pigmented colonies were further confirmed to be *E. sakazakii* using the API 20E test system. Finally, the presence of this microbe in wine sample RW1 was confirmed by the PCR amplification reaction specific for the detection of *E. sakazakii* (Keyser *et al*., 2003) during which both the wine sample DNA and the positive control showed to have a single PCR band of fragment size 850 bp (results not shown). *Enterobacter sakazakii* is a member of the *Enterobacteriaceae* family, an emerging pathogen and has been the cause of illnesses and deaths in infants. Although it has been associated with contaminated infant formulas, it has also been isolated from ready- to-eat foods, raw vegetables and unpasteurised fruit and vegetable



a, b, c, d, e, f, g – DGGE bands identified using DNA sequencing

Figure 1 PCR-based DGGE analysis of a spoilt South African red wine (RW1) using *Bacteria* specific primers.



h – DGGE band identified using DNA sequencing

Figure 2 PCR-based DGGE analysis of a spoilt South African red wine (RW1) using yeast specific primers.

juices (Kim & Beuchat, 2005). Although not commonly associated with wine, this microbe could have entered the wine fermentation by contamination from contact with the soil, the irrigation water or from the grape surface and survived throughout the fermentation and aging processes.

PCR-based DGGE analysis using yeast specific primers (Fig. 2) shows no clear difference in concentration between the unfiltered and filtered wine samples in the DGGE profile. Only one band could be identified in this profile. Band h was identified as *S. cerevisiae* (98% homology, 176 out of 178 bases) (GenBank Accession number AB212636). This wine was confirmed to be spoilt by *S. cerevisiae* using conventional plating by the Department of Viticulture and Oenology, Stellenbosch University.

RW2

PCR-based DGGE analysis (Fig. 3) showed that five bands were present from the PCR amplification using *Bacteria* specific primers and one band using yeast specific primers. Band i was identified as an uncultured bacterial clone 21BSF28 (91% homology, 130 out of 143 bases) (GenBank Accession number AJ863280), closely related to *Pseudomonas* sp. These microbes are Gram-negative rods, non-fermentative, grow over a wide temperature range (4°C - 43°C) and isolated from soil and plants. They are strictly aerobic, therefore their growth in wine is unlikely because of the anaerobic conditions of the winemaking process (Krieg & Holt, 1984). The presence of this microbe in the wine sample could be due to contamination of the wine when the sample was taken.

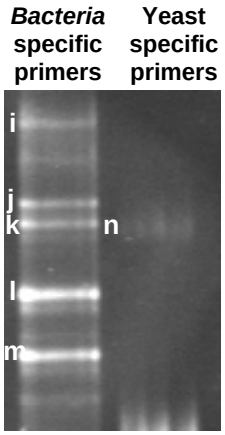
Both band j (99% homology, 160 out of 161 bases) (GenBank Accession number AJ852327) and band l (96% homology, 151 out of 157 bases) (GenBank Accession number AJ852327) were identified as an uncultured bacterial clone MKEL-242, closely related to *P. agglomerans*. Again, interspecies heterogeneity could lead to the detection of several bands when only one species is present (Coenye & Vandamme, 2003).

Band k was identified as an uncultured bacterial clone PBg1-024 (99% homology, 158 out of 159 bases) (GenBank Accession number AY791163), closely related to *E. sakazakii*. Band m was identified as an uncultured bacterial clone BPH1C14003 (100%

homology, 150 out of 150 bases) (GenBank Accession number DQ221308), closely related to *E*. *sakazakii*. By comparison of the results from the PCR-based DGGE technique and the conventional plating method of the spoilt wine sample on the different media, none of the above mentioned microbes were isolated from the media. However, the sample was not enriched for *E. sakazakii* isolation and the concentration of *E. sakazakii* could therefore have been too low to isolate using conventional plating. Also, the identification of members of the *Enterobacteriaceae* by PCR-based DGGE and not by conventional plating could be due to the composition of the media used since the media was not specific for *E. sakazakii*.

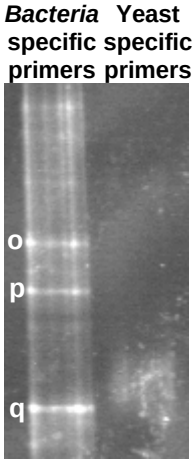
Only one bacterial species could be identified on the selective media tested. This microbe was identified as *Acetobacter pasteurianus* (97% homology, 396 out of 405 bases) (GenBank Accession number AY883035) at a concentration of 67 x 10^{-1} cfu.ml^{-1}. Although it was long believed that AAB are obligate aerobes and that growth was not possible given the anaerobic conditions of the winemaking process, it has been shown that certain species of AAB, one of which is *A*. *pasteurianus*, can continue to grow during alcoholic fermentation, MLF and the maturation of the wine (Du Toit & Lambrechts, 2002; Joyeux *et al*., 1984; Drysdale & Fleet,1988). Therefore, this microbe could have been the cause of spoilage in this red wine. The fact that this microbe was not identified by PCR-based DGGE could be explained by too low cell numbers in the wine sample (Ercolini, 2004; Savazzini & Martinelli, 2005).

This wine was confirmed to be spoilt by *S. cerevisiae* using conventional plating by the Department of Viticulture and Oenology, Stellenbsoch University. This was confirmed by PCR-based DGGE where band n was identified as *S*. *cerevisiae* (99% homology, 176 out of 178 bases) (GenBank Accession number AB212636) and by sequencing the isolate from the YPD plates (100% homology, 186 out of 186 bases) (GenBank Accession number AY601161).



i, j, k, l, m, n – DGGE bands identified using DNA sequencing

Figure 3 PCR-based DGGE analysis of a spoilt South African red wine (RW2).



o, p, q – DGGE bands identified using DNA sequencing

Figure 4 PCR-based DGGE analysis of a spoilt South African red wine (RW3).

RW3

PCR-based DGGE analysis (Fig. 4) showed that three bands were present from the PCR amplification using *Bacteria* specific primers and no bands using yeast specific primers. Band o was identified as an uncultured bacterial clone MKEL-242 (96% homology, 151 out of 157 bases) (GenBank Accession number AJ852327), closely related to *P. agglomerans*. Band p was identified as an uncultured bacterial clone BPH1C14003 (100% homology, 150 out of 150 bases) (GenBank Accession number DQ221308), closely related to *E. sakazakii*. Band q was presumptively identified as *Acetobacter* sp. CGDNIH1 (95% homology, 72 out of 76 bases) (GenBank Accession number AY788950). *Acetobacter* spp. that are regarded as spoilage microbes in wine are *A. pasteurianus*, *Acetobacter aceti*, *Gluconacetobacter liquefaciens* and *Gluconacetobacter hansenii* (Du Toit & Lambrechts, 2002) and these species can tolerate concentrations of 10 - 15% (v/v) ethanol (Drysdale & Fleet, 1988), which can explain its presence in the bottled wine. This wine had a bitter taint which is a well-known defect in spoilt wines. This defect can be the result of glycerol catabolism in red wine (Sponholz, 1993). The ability to degrade glycerol is not common amongst LAB, where only 31% of *Lactobacillus* spp. have shown to have this ability (Sponholz, 1993). However, the “bitter” taint could have been mistaken for a high concentration of acetaldehyde which gives an oxidized flavour to wine (Drysdale & Fleet, 1989). This compound could have been produced by the *Acetobacter* sp. identified in the wine by PCR-based DGGE resulting in spoilage. The ability of AAB to reach a viable but non-culturable (VBNC) state has been studied (Millet & Lonvaud-Funel, 2000). The fact that *Acetobacter* sp. was identified using PCR-based DGGE and not isolated by using selective plating, confirms that these microbes are difficult to culture and that the conditions was not suitable for its growth, suggesting a VBNC state.

No bands could be identified by PCR-based DGGE using the yeast specific primers. By comparison of the PCR-based DGGE technique with that of the microbial cultivation and plating, no growth was observed on any of the four selective media.

RoW1

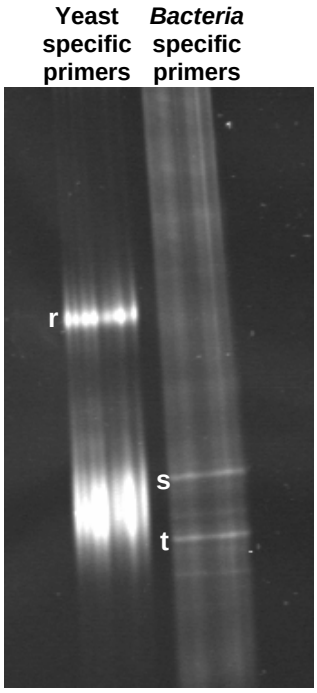
PCR-based DGGE analysis (Fig. 5) showed that one band was present using yeast specific primers. Band r was identified as *S. cerevisiae* (99% homology, 206 out of 207 bases) (GenBank Accession number AJ870460). This wine was confirmed to be spoilt by *S. cerevisiae* using conventional plating by the Department of Viticulture and Oenology, Stellenbosch University.

Using *Bacteria* specific primers, band s (99% homology, 149 out of 151 bases) (GenBank Accession number AY376705) was identified as an uncultured bacterial clone O6 closely related to *T. guamensis* and could possibly have added to the spoilage of the wine. Band t could not be identified as bacterial DNA and may be due to contamination of the wine sample from other sources.

WW1

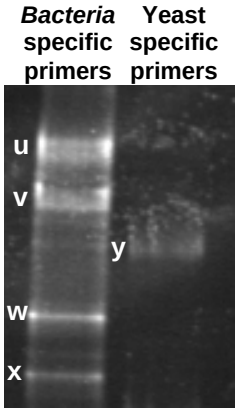
PCR-based DGGE analysis (Fig. 6) showed four bands were present from the PCR amplification using *Bacteria* specific primers and one band using yeast specific primers. Band u was identified as an uncultured bacterial clone 21BSF28 (91% homology, 130 out of 143 bases) (GenBank Accession number AJ863280), closely related to *Pseudomonas* sp. Band v was presumptively identified as a gamma proteobacterium Ga-40 (98% homology, 49 out of 50 bases) (GenBank Accession number AJ561194), closely related to *Pseudoalteromonas* sp. These microbes are members of the family *Pseudomonadaceae* (Krieg & Holt, 1984) and their presence in the wine could be due to contamination of the wine sample, since these microbes are unlikely to be associated with wine.

Band w was identified as *P. agglomerans* (95% homology, 154 out of 162 bases) (GenBank Accession number AJ852057). Band x was identified as an uncultured bacterial clone WS05A-G02 (94% homology, 141 out of 150 bases) (GenBank Accession number DQ171138), closely related to *Citrobacter farmeri*. This microbe is a member of the family *Enterobacteriaceae* and is isolated from soil, water and food. It is a facultative anaerobic, fermentative microbe and could have entered the wine fermentation at the time of harvesting (Krieg & Holt, 1984).



r, s, t – DGGE bands identified using DNA sequencing

Figure 5 PCR-based DGGE analysis of a spoilt South African rosé wine (RoW1).



u, v, w, x, y – DGGE bands identified using DNA sequencing

Figure 6 PCR-based DGGE analysis of a spoilt South African white wine (WW1).

By comparison of the results from the PCR-based DGGE technique and the conventional plating method of the spoilt white wine sample on the different media, none of the above mentioned microbes were isolated from the media and only one bacterial species could be identified. This microbe was identified as *Lactobacillus* sp. KC38 (98% homology, 259 out of 265 bases) (GenBank Accession number AF243160). *Lactobacillus* species are commonly found on grapes, in the must and wine (Fugelsang, 1997). The fact that this microbe could not be identified by PCR-based DGGE could be explained by the possibility of a too low concentration (Ercolini, 2004, Savazzini & Martinelli, 2005).

This wine was confirmed to be spoilt by *S. cerevisiae* using conventional plating by the Department of Viticulture and Oenology, Stellenbosch University and this was confirmed by PCR-based DGGE when band y was identified as *S*. *cerevisiae* (99% homology, 176 out of 178 bases) (GenBank Accession number AB212636). This was also confirmed when *S*. *cerevisiae* was isolated and identified from the YPD plates (99% homology, 188 out of 189 bases) (GenBank Accession number AB212636). These results show that there is a correlation between PCR-based DGGE and conventional plating and that PCR-based DGGE can be used as an alternative to selective plating for the identification of spoilage yeasts.

Conclusions

Five spoilt commercial wine samples were analysed using PCR-based DGGE to identify the microbes responsible for the spoilage. RW1, RW2 and WW1 were known to be spoilt by the yeast *S. cerevisiae* and this was confirmed by PCR-based DGGE. This microbe was also isolated from RW2 and WW1 using conventional plating. RoW1 was spoilt by an unknown microbe, and *S. cerevisiae* was found to be present in the wine by PCR-based DGGE. Other microbes detected in these wine samples were members of the family *Enterobacteriaceae* and uncultured bacteria which could also possibly add to the spoilage of wines. In RW3 an *Acetobacter* sp. was identified using PCR-based DGGE which could have been the cause of the spoilage.

Compared to conventional microbiological methods, the presence of members of the family *Enterobacteriaceae* was confirmed in RW1. Other microbes isolated from the samples by plating were *Lactobacillus* sp. in WW1 and *A. pasteurianus* in RW2. No growth was observed in RW3.

Microbiological plating has been used to identify spoilage yeasts, LAB and AAB from wine (Ubeda & Briones, 1999). However, these plating methods are time-consuming and not all microbes can be cultured on synthetic growth media (Heard & Fleet, 1986; Kopke *et al*., 2000). PCR-based DGGE have been used successfully as a culture–independent method for the fingerprinting of the microbial diversity in wine (Cocolin *et al*., 2000; Kawai *et al*., 2002; Lopez *et al*., 2003; Prakitchaiwattana *et al*., 2004). The results from this study indicated that PCR-based DGGE proved to be a possible alternative to be used in conjunction with conventional microbiological methods for the detection of spoilage microbes in wine.

References

Altschul, S.F., Madden, T.L. Schaffer, A.A., Zhang, Z., Miller, W. & Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acid Research*, **25**, 3389-3402.

Coenye, T. & Vandamme, P. (2003). Intragenomic heterogeneity between multiple 16S ribosomal RNA operon in sequenced bacterial genomes. *FEMS Microbiology Letters*, **228**, 45-48.

Cocolin, L., Bisson, L.F. & Mills, D.A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiology Letters*, **189**, 81-87.

Du Toit, W.J. & Lambrechts, M.G. (2002). The enumeration and identification of acetic acid bacteria from South African red wine fermentations. *International Journal of Food Microbiology*, **74**, 57-64.

Drysdale, G.S. & Fleet, G.H. (1988). Acetic acid bacteria in winemaking: a review. *American Journal of Enology and Viticulture*, **39**, 143-152.

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, **56**, 297-314.

Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E. & Akkermans, A.D.L. (1997). Ribosome analysis reveals prominent activity of an uncultured member of the class *Acinetobacteria* in grassland soils. *Microbiology*, **143**, 2983-2989.

Fleet, G.H. (2003). Yeast interactions and wine flavour. *International Journal of Food Microbiology*, **86**, 11-22.

Fugelsang, K.C. (1997). Wine Microbiology. Pp. 3-47, 48-67, 68-111, 132-137. New York: Chapman & Hall.

Gavini, F., Mergaert, J., Beiji, A., Mielcarek, C., Izard, D., Kersters, K. & De Ley, J. (1989). Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov. *International Journal of Systematic Bacteriology*, 39, 337-345.

Heard, G.M. & Fleet, G.H. (1986). Evaluation of selective media for enumeration of yeasts during wine fermentation. *Journal of Applied Bacteriology*, **60**, 477-481.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. & Williams, S.T. (1994). Bergey's Manual of Determinative bacteriology, 9th ed. Pp. 175, 178, 184. Maryland: Williams & Wilkens.

Joyeux, A., Lafon-Lafourcade, S. & Ribèreau-Gayon, P. (1984). Evolution of acetic acid bacteria during fermentation and storage of wine. *Applied and Environmental Microbiology*, **48**, 153-156.

Keyser, M., Witthuhn, R.C., Ronquest, L.C. & Britz, T.J. (2003). Treatment of winery effluent with upflow anaerobic sludge blanket (UASB) – granular sludges enriched with *Enterobacter sakazakii*. *Biotechnology Letters*, **25**, 1893-1898.

Kim, H. & Beuchat, L.R. (2005). Survival and growth of *Enterobacter sakazakii* on fresh-cut fruits and vegetables and in unpasteurised juices as affected by storage temperature. *Journal of Food Protection*, **68**, 2541-2552.

Kopke, C., Cristovão, A., Prata, A.M., Silva Pereira, C., Figueiredo Marques, J.J. & San Romão, M.V. (2000). Microbiological control of wine. The application of

epfiluorescence microscopy method as a rapid technique. *Food Microbiology*, **17**, 257-260.

Krieg, N.R. & Holt, J.G. (1984). Bergey's Manual of Systematic bacteriology, Vol. 1. Pp. 141, 408 – 409, 585. London: Williams & Wilkens.

Lambrechts, M.G. & Pretorius, I.S. (2000). Yeast and its importance to wine aroma – a review. *South African Journal of Enology and Viticulture*, **21**, 97-129.

Loureiro, V. & Malfeito-Ferreira, M. (2003). Spoilage yeasts in the wine industry. *International Journal of Food Microbiology*, **86**, 23-50.

McWhorter, A.C., Haddock, R.L., Nocon, F.A., Steigerwalt, A.G., Brenner, D.J., Aleksić, S., Bockemühl, J. & Farmer, J.J. (1991). *Trabulsiella guamensis*, a new genus and species of the family *Enterobacteriaceae* that resembles *Salmonella* subgroups 4 and 5. *Journal of Clinical Microbiology*, **29**, 1480-1485.

Millet, V. & Lonvaud-Funel, A. (2000). The viable but non-culturable state of wine micro-organisms during storage. *Letters in Applied Microbiology*, **30**, 136-141.

Muyzer, G., de Waal, E.C. & Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, **59**, 695-700.

O'Donnell, K. (1993). Fusarium and its near relatives, In: *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics* (edited by Reynolds, D.R. & Taylor, J.W.). Pp. 225-233. Wallingford, UK: CAB International.

Querol, A., Fernández-Espinar, M.T., lí del Olmo, M. & Barrio, E. (2003). Adaptive evolution of wine yeast. *International Journal of Food Microbiology*, **86**, 3-10.

Rapp, A. & Versini, G. (1991). Influence of nitrogen compounds in grapes on aroma compounds in wine. In: *Proceedings of the International symposium on nitrogen in grapes and wines* (edited by RANTZ). Pp. 156-164. Davis, CA: American Society for Enology and Viticulture.

Romano, P., Fiore, C., Paraggio, M., Caruso, M. & Capece, A. (2003). Function of yeast species and strains in wine flavour. *International Journal of Food Microbiology*, **86**, 169-180.

Savazzini, F. & Martinelli, L. (2006). DNA analysis in wines: Development of methods for enhanced extraction and real-time polymerase chain reaction quantification. *Analytica Chimica Acta*, **563**, 274-282.

Sponholz, W-R. (1993). Wine Spoilage by Microorganisms. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 395-420. New York: Taylor & Francis.

The South African Wine Laboratories Association. (2002). *Methods of analysis for wine laboratories*. South Africa: South African Society for Enology and Viticulture.

Van Elsas, J.D., Matynen, V. & Wolters, A. (1997). Soil DNA extraction and assessment of the fate of *Mycobacterium chlorophenolicum* strain PCR-1 in different soils by 16S ribosomal RNA gene sequence based most-probable-number PCR immunofluorensence. *Biological Fertilization*, **24**, 188-195.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Winemaking involves the interaction between a variety of yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and mycelial fungi and the metabolic by-products of these microbes can influence the wine either positively or negatively. Therefore, to ensure wines of a good quality it is important to identify the microbes present during the winemaking process (Rapp & Versini, 1991; Fleet, 1993; Du Toit & Pretorius, 2000; Lambrechts & Pretorius, 2000; Fleet, 2003). Although conventional microbiological platings have been used to isolate microbes from wine, these methods proved to be time-consuming and certain microbes do not grow on synthetic isolation media (Heard & Fleet, 1986; Kopke *et al*., 2000).

Polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) is a non-culturable approach and was used to identify the microbes present throughout the fermentation processes of two South African red wines, Pinotage and Merlot, as well as five spoilt commercial wines. The results were compared to conventional plating methods.

Using yeast specific primers, analysis of Pinotage and Merlot wines during the alcoholic fermentation and malo-lactic fermentation (MLF) showed that the fermentations were carried out by complex microbial populations which consist of a succession of yeast species. The Pinotage wine showed the presence of non-*Saccharomyces* yeasts at the beginning of the alcoholic fermentation, while *Saccharomyces cerevisiae* was present until the completion of the MLF. This yeast was also isolated during both the alcoholic fermentation and MLF of the Merlot wine using PCR-based DGGE and conventional plating. However, the presence of *S. cerevisiae* in bottled wine could lead to spoilage (Fleet, 2003). This yeast proved to be the cause of spoilage in four of the five spoilt commercial wine samples (RW1, RW2, RoW1 and WW1) and this was confirmed using conventional plating.

Using *Bacteria* specific primers, *Lactobacillus plantarum* and *Lactobacillus* sp. were identified in the Pinotage wine using PCR-based DGGE, while *Lactobacillus brevis* was isolated from Merlot wine and *Lactobacillus* sp. from WW1 using

conventional platings. The fact that these microbes could not be detected by PCR-based DGGE could be explained by a too low concentration of the microbes in the wine or that the polysaccharides, tannins and polyphenols in the wine inhibited DNA extraction (Ercolini, 2004, Savazzini & Martinelli, 2005). Of the AAB, an *Acetobacter* sp. was identified in RW3 using PCR-based DGGE, but not by conventional plating. This confirms that these microbes are difficult to culture and that the conditions were not suitable for growth (Du Toit & Pretorius, 2002). However, *Acetobacter pasteurianus* could be isolated from RW2 using conventional plating, indicating suitable conditions for its growth.

In all the wine samples, members of the family *Enterobacteriaceae*, such as the opportunistic pathogen, *Enterobacter sakazakii* and *Pantoea agglomerans* were identified. These microbes are not commonly associated with wine, however, they are ubiquitous in nature and have been isolated from soil, water, seeds, fruit and plant surfaces (Gavini *et al*., 1989). Therefore, the grapes could have been contaminated during irrigation, harvesting or transport. Since grapes are not washed prior to fermentation, these microbes could have entered the fermenting wines. The presence of *E. sakazakii* was also confirmed in RW1 by conventional plating, using the API 20E test system and by an *E. sakazakii* detection PCR reaction.

Concluding remarks

The results indicated that PCR-based DGGE is an effective alternative technique to directly characterise the yeast and bacteria diversity in red grape must and wine, as well as the identification of spoilage microbes in spoilt commercial wines. Compared to conventional microbiological methods, PCR-based DGGE proved to be a rapid and reliable alternative. Future studies could include in depth studies on the use of PCR-based DGGE for the identification of spoilage microbes in wines, as well as the comparison between molecular techniques and conventional cultivation and isolation.

The filtration of the samples prior to DNA isolation improved the visibility of the bands in the PCR-based DGGE profile possibly due to a higher concentration of the microbial cells or by eliminating some of the compounds that may inhibit DNA extraction. However, it was clear from this study that DNA extraction is an important

parameter in PCR-based DGGE and therefore future studies could include the comparison of alternative methods of DNA extraction. Also, this study proved the concentration of DNA used in PCR reactions are extremely important for successful PCR reactions and future studies could focus on the standardisation of the concentrations used in PCR reactions.

The isolation of members of the family *Enterobacteriaceae* from the wine samples in this study can lead to important future studies of the growth and survival of these microbes in the wine environment. Since these microbes are not commonly tested for their presence in wines, the techniques used in this study could be used for future studies on *Enterobacteriaceae* in wines.

This study also revealed the identification of several bands of microbes when only one species was present. This is due to the interspecies heterogeneity of the 16S rRNA gene (Coenye & Vandamme, 2003). A possible solution to the problem could be to target the RNA polymerase beta subunit gene *rpoB* in future studies (Dahllöf *et al*., 2000).

References

Coenye, T. & Vandamme, P. (2003). Intragenomic heterogeneity between multiple 16S ribosomal RNA operon in sequenced bacterial genomes. *FEMS Microbiology Letters*, **228**, 45-48.

Dahllöf, I., Baillie, H. & Kjelleberg, S. (2000). *rpoB*-Based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. *Applied and Environmental Microbiology*, **66**, 3376-3380.

Du Toit, M. & Pretorius, I.S. (2000). Microbial spoilage and preservation of wine: using weapons from nature's own arsenal – a review. *South African Journal of Enology and Viticulture*, **21**, 74-96.

Du Toit, W.J. & Pretorius, I.S. (2002). The occurrence, control and esoteric effect of acetic acid bacteria in winemaking. *Annals of Microbiology*, **52**, 155-179.

Ercolini, D. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, **56**, 297-314.

Fleet, G.H. (1993). The microorganisms of winemaking – isolation, enumeration and identification. In: *Wine Microbiology & Biotechnology* (edited by G.H. Fleet). Pp. 1-26. New York: Taylor & Francis.

Fleet, G.H. (2003). Yeast interactions and wine flavour. *International Journal of Food Microbiology*, **86**, 11-22.

Gavini, F., Mergaert, J., Beiji, A., Mielcarek, C., Izard, D., Kersters, K. & De Ley, J. (1989). Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov. *International Journal of Systematic Bacteriology*, 39, 337-345.

Heard, G.M. & Fleet, G.H. (1986). Evaluation of selective media for enumeration of yeasts during wine fermentation. *Journal of Applied Bacteriology*, **60**, 477-481.

Kopke, C., Cristovão, A., Prata, A.M., Silva Pereira, C., Figueiredo Marques, J.J. & San Romão, M.V. (2000). Microbiological control of wine. The application of epfiluorescence microscopy method as a rapid technique. *Food Microbiology*, **17**, 257-260.

Lambrechts, M.G. & Pretorius, I.S. (2000). Yeast and its importance to wine aroma – a review. *South African Journal of Enology and Viticulture*, **21**, 97-129.

Rapp, A. & Versini, G. (1991). Influence of nitrogen compounds in grapes on aroma compounds in wine. In: *Proceedings of the International symposium on nitrogen in grapes and wines* (edited by RANTZ). Pp. 156-164. Davis, CA: American Society for Enology and Viticulture.

Savazzini, F. & Martinelli, L. (2006). DNA analysis in wines: Development of methods for enhanced extraction and real-time polymerase chain reaction quantification. *Analytica Chimica Acta*, **563**, 274-282.