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

Michelle CAMERON[1], Leoni SIEBRITS[1], Maret du TOIT[2] and Corli WITTHUHN[1,*,#]

1 : Department of Food Science, Stellenbosch University, Private Bag X1, Matieland (Stellenbosch) 7602, South Africa

2 : Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University, Private Bag X1, Matieland (Stellenbosch) 7602, South Africa

Abstract

Aim : The aim of this study was to evaluate the microbial population present in red grape must and wine using polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE).

Methods and results : Red wine from the cultivars Pinotage and Merlot were produced and samples taken throughout alcoholic and malolactic fermentation (MLF). PCR fragments were resolved by DGGE and unique fingerprints were obtained for the bacteria and yeasts present in the wines. *Lactobacillus plantarum*, *Enterobacter sakazakii* (*Cronobacter* sp.) and *Pantoea agglomerans* were present in the Pinotage during both alcoholic and MLF, and in both inoculated and spontaneous fermentations. *E. sakazakii* (*Cronobacter* sp.) and *P. agglomerans* were also observed in the Merlot wines during alcoholic fermentation as well as MLF. *Saccharomyces cerevisiae* was the most dominant yeast observed in Pinotage, and was the only yeast observed in Merlot. This yeast was observed until the end of MLF.

Conclusions : Results showed that the microbial flora that participates in the winemaking process is more diverse than commonly thought.

Significance and impact of the study : This method may serve as an alternative to conventional microbiological methods for the identification of the microbial species in red grape must and wine.

Key words : red wine, alcoholic fermentation, malolactic fermentation, *Enterobacter sakazakii* (*Cronobacter* sp.), *Pantoea agglomerans*

Résumé

Objectif : Le but de cette étude était d'évaluer la population microbienne présente dans le moût et le vin de raisin rouge, en utilisant la réaction en chaîne par polymérase (PCR) couplée à l'électrophorèse sur gel en gradient dénaturant (DGGE).

Méthodes et résultats : Du vin rouge des cultivars Pinotage et Merlot ont été produits et des échantillons ont été prélevés pendant toute la fermentation alcoolique et malolactique (FML). Des fragments PCR ont été résolus par DGGE et des bandes uniques ont été obtenues pour les bactéries et les levures présentes dans les vins. *Lactobacillus plantarum*, *Enterobacter sakazakii* (*Cronobacter* sp.) et *Pantoea agglomerans* étaient présentes dans le Pinotage au cours des deux fermentations alcooliques et FML, à la fois dans les fermentations provoquées et spontanées. *E. sakazakii* (*Cronobacter* sp.) et *P. agglomerans* ont également été observées dans les vins Merlot, pendant la fermentation alcoolique ainsi que la FML. *Saccharomyces cerevisiae* était la levure la plus dominante observée dans le Pinotage, et l'unique levure observée dans le Merlot. Cette levure a été observée jusqu'à la fin de la FML.

Conclusions : Les résultats ont montré que la flore microbienne qui participe au processus de vinification est plus diversifiée qu'on ne le croit généralement.

Importance et impact de l'étude : Cette méthode peut servir d'alternative aux méthodes microbiologiques conventionnelles utilisées pour l'identification des espèces microbiennes dans le moût et le vin de raisin rouge.

Mots clés : vin rouge, fermentation alcoolique, fermentation malolactique, *Enterobacter sakazakii* (*Cronobacter* sp.), *Pantoea agglomerans*

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*Corresponding author : witthuhnrc@ufs.ac.za

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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 fermentation steps, the initial alcoholic fermentation, followed by malolactic fermentation (MLF) (Boulton *et al*., 1996). During 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 and Versini, 1991 ; Fleet and Heard, 1993). Alcoholic fermentation can take place either 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 and Heard, 1993 ; Fugelsang, 1997). The non-*Saccharomyces* yeasts that initiate alcoholic fermentation include species of the genera *Hanseniaspora*, *Candida*, *Metschnikowia*, *Hansenula*, *Zygosaccharomyces*, *Brettanomyces*, *Aureobasidium*, *Rhodotorula*, *Pichia*, *Kluyveromyces*, *Cryptococcus*, *Dekkera*, *Schizosaccharomyces*, *Torulaspora* and *Saccharomycodes* (Fleet and 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 *Lactobacillaceae*, characterised by the genera *Lactobacillus* and *Pediococcus*, and the *Leuconostocaceae*, characterised by the genera *Leuconostoc* and *Oenococcus* (Henick-Kling, 1993 ; Fugelsang, 1997). *Pediococcus damnosus*, *Leuconostoc mesenteroides*, *Lactobacillus plantarum* and *Oenococcus oeni* have been identified as the key LAB accountable for MLF (Boulton *et al*., 1996 ; 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 and Fleet, 1986 ; Kopke *et al*., 2000). Molecular techniques offer new opportunities for identifying all the species present in a population (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). The limitation of this method is that detection levels are 10^2-10^4 cfu/mL depending on the probe and matrix, high yields of DNA of all the species present are required, and some wine compounds can inhibit the DNA extraction and PCR reaction (Cocolin *et al*., 2000 ; Fernández-González *et al*., 2001 ; Ercolini, 2004 ; Prakitchaiwattana *et al*., 2004). Furthermore, a single species with multiple ribosomal RNA (rRNA) copies can portray multiple bands in the DGGE profile, overestimating the diversity of the microbes in the sample.

The aim of this study was to identify the microbial population present in South African red grape must and wine by using PCR-based DGGE fingerprinting and DNA sequencing.

MATERIALS AND METHODS

1. 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 alcoholic fermentation on day six. The inoculated alcoholic fermentation was also inoculated with *O. oeni* after the completion of alcoholic fermentation, while the spontaneous fermentation was left to undergo spontaneous MLF, both for a duration of seven 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 two days at 15 °C, after which it was inoculated with *S. cerevisiae* WE14. Alcoholic fermentation was carried out at 25 °C and 50 mL samples were taken daily until completion on day nine. After the completion of the alcoholic fermentation, the wine was left to undergo spontaneous MLF for eleven weeks and 50 mL samples were taken weekly.

2. DNA isolation

DNA was extracted from 2 mL of each sample of the Pinotage and Merlot must and wine. Prior to

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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 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 60ºC. This step was repeated twice. After incubation, the samples were 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: iso-amylalcohol (25: 24: 1) mixture and repeated until the interphase was clear. 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).

3. PCR-based DGGE analysis

The 5' end of the V3 variable region of the 16S rRNA gene was amplified using the bacteria specific primers F341^{GC} (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 an Eppendorf Mastercycler Personal. An initial 4 min denaturation at 94ºC was followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 54ºC for 60 s and elongation at 72ºC for 60 s and a final 5 min chain elongation at 72ºC (Muyzer *et al*., 1993).

The 5' end of the 26S rRNA gene was amplified using the yeast specific primers NL1^{GC} (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 from the Merlot samples were used. The DNA was amplified during 30 cycles of denaturation at 95ºC for 60 s, annealing at 52ºC for 45 s and elongation at 72ºC for 60 s. An initial 5 min denaturation at 95ºC and a final 7 min chain elongation at 72ºC were performed (Cocolin *et al*., 2000).

The PCR fragments were separated using DGGE, performed with the 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 bacteria 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 ºC. The gels were stained in 1 x TAE buffer containing ethidium bromide (94 $\mu L.L^{-1}$) and the fragments were visualized under UV light (Vilber Lourmat).

4. 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 bacteria 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 SigmaSpin Post-Reaction Clean-up 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).

RESULTS AND DISCUSSION

1. DGGE fingerprinting

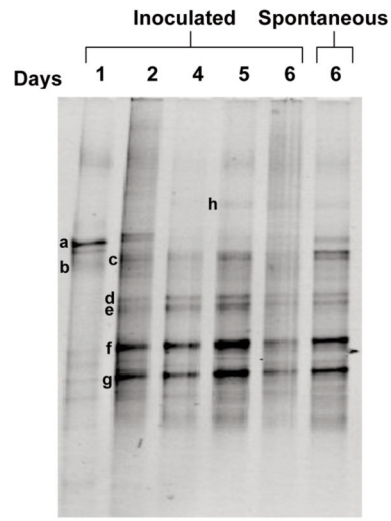
a. Pinotage wine

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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 (Figure 1) showed that the DGGE profile changed over the six-day fermentation period and that the DGGE fingerprint of day six of spontaneous alcoholic fermentation was similar to that of day six of the inoculated fermentation. The profile during spontaneous MLF was the same as for day six of inoculated and spontaneous alcoholic fermentation (data not shown).

Of the eight bands observed, band a disappeared after one day of inoculated alcoholic fermentation (Figure 1) and was identified as *Lactobacillus plantarum* (100 % homology) (GenBank Accession number AY383631) (Table 1). Band b was present throughout the alcoholic fermentation (Figure 1) and MLF processes and was identified as *Lactobacillus* sp. clone A12-10c (99 % homology) (GenBank Accession number DQ056428), closely related to *L. plantarum* (Table 1). *L. plantarum*, as well as other *Lactobacillus* species are commonly found on grapes, in the must and wine (Fugelsang, 1997). However, certain *L. plantarum* strains are not tolerant of high ethanol or SO_2 concentrations (Henick-Kling, 1993 ; G-Alegría *et al*., 2004), 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 (Table 1). Although these bands are not clearly visible, visual inspection of the gel under the UV light showed these bands



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.

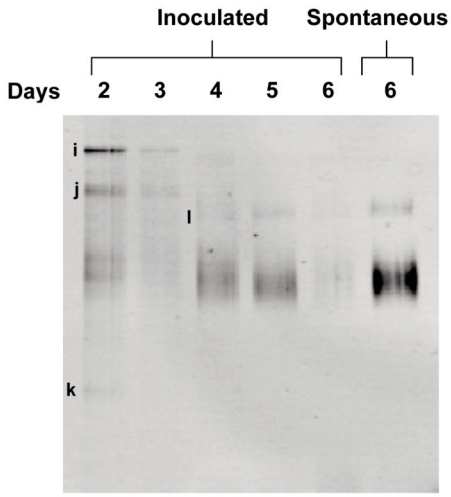
Table 1 - A summary of the different bacteria and yeasts identified at different stages during Pinotage production.

	Alcoholic fermentation (days)						
	1	2	3	4	5	6	
Inoculated	a, b, i, j, k	b, c, d, e, f, g, i, j, k	b, c, d, e, f, g, i, j	b, c, d, e, f, g, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	
Spontaneous	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	
	Malolactic fermentation (weeks)						
	1	2	3	4	5	6	7
Inoculated	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l
Spontaneous	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l	b, c, d, e, f, g, h, l

a : *Lactobacillus plantarum*, b : *Lactobacillus* sp., c : uncultured bacterial clone (*Enterobacter sakazakii*) (*Cronobacter* sp.), d : *Enterobacter* sp. (*Enterobacter sakazakii*) (*Cronobacter* sp.), e : uncultured bacterial clone (*Enterobacter sakazakii*) (Cronobacter sp.), f : *Pantoea agglomerans*, g : uncultured bacterial clone (*Pantoea agglomerans*), h : uncultured bacterial clone (*Enterobacter sakazakii*) (*Cronobacter* sp.), i : *Hanseniaspora uvarum*, j : *Zygosaccharomyces rouxii*, k : *Issatchenkia orientalis*, l : *Saccharomyces cerevisiae*

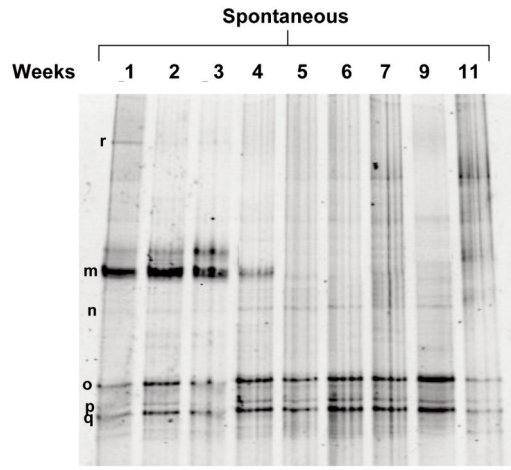
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were present. These were punched from the gel to confirm results by sequencing. Band c (96 % homology) (GenBank Accession number DQ171118) was identified as an uncultured bacterial clone WS05A_D12 closely related to *Enterobacter sakazakii*. Band d was identified as *Enterobacter* sp. (100 % homology) (GenBank Accession number AY576743), closely related to *E. sakazakii* and band e (100 % homology) (GenBank Accession number AB234526) was identified as an uncultured bacterial clone MgMjD-114 closely related to *E. sakazakii*. Band f was identified as *P. agglomerans* (100 % homology) (GenBank Accession number AY315454). Band g (99 % homology) (GenBank Accession number AY186083) was identified as an uncultured bacterial clone LB1B7 closely related to *P. agglomerans* and band h (99 % homology) (GenBank Accession number AY376705) was identified as an uncultured bacterial clone O6 closely related to *E. sakazakii*. These species are members of the family *Enterobacteriaceae* and are not commonly associated with wine. However, Ruiz *et al*. (2010) recently reported the presence of *Enterobacter* sp. in fermented grape must during early, middle and late MLF. Similarly, Nisiotou *et*



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

Figure 2 - 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 the inoculated fermentation period. Lane 6 : day six of the spontaneous fermentation.



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

Figure 3 - PCR-based DGGE analysis of South African Merlot must and wine samples during malolactic fermentation (MLF) using bacteria specific primers. Lanes 1 - 9 : weeks one to seven, nine and eleven of spontaneous MLF.

Table 2 - A summary of the different bacteria and yeasts identified at different stages during Merlot production.

Inoculated alcoholic fermentation (days)										
1	2	3	4	5	6	7	8	9		
m, n, o, p, q, s	m, n, o, p, q, s	m, n, o, p, q, s	m, n, o, p, q, s	m, n, o, p, q, r, s	m, n, o, p, q, r, s	m, n, o, p, q, r, s	m, n, o, p, q, r, s	m, n, o, p, q, r, s		
Spontaneous malolactic fermentation (weeks)										
1	2	3	4	5	6	7	8	9	10	11
m, n, o, p, q, r, s	m, n, o, p, q, r, s	m, n, o, p, q, r, s	m, n, o, p, q, s	m, n, o, p, q, s	n, o, p, q, s	n, o, p, q, s	n, o, p, q, s	n, o, p, q, s	o, p, q, s	o, p, q, s

m : uncultured bacterial clone, n : *Enterobacter sakazakii* (*Cronobacter* sp.), o : *Pantoea agglomerans*, p : *Enterobacter sakazakii* (*Cronobacter* sp.), q : uncultured bacterial clone (*Pantoea agglomerans*), r : uncultured bacterial clone (*Pantoea agglomerans*), s : *Saccharomyces cerevisiae*

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al. (2011) reported the presence of *Enterobacter* sp. on grape surfaces and in grape must, and the presence of *P. agglomerans* in ice-wine must has also been reported (Subden *et al*., 2003). 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, as well as with the water used in the cellar environment. Since grapes are not washed prior to fermentation, these microbes could have entered the fermenting wine.

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 (Figure 2) showed that the profiles changed significantly during the course of the fermentation process. The DGGE fingerprint of day six of spontaneous alcoholic fermentation was similar to that of day six of the inoculated fermentation. The DGGE profile during spontaneous MLF showed fewer bands than the alcoholic fermentation profile. Band i was identified as *Hanseniaspora uvarum* (99 % homology) (GenBank Accession number HUU84229) and band j as *Zygosaccharomyces rouxii* (95 % homology) (GenBank Accession number AJ966531). These two microbes died-off after three days of alcoholic fermentation (Figure 2, Table 1). Band k was identified as *Issatchenkia orientalis* (100 % homology) (GenBank Accession number AY601160) which died-off after two days of alcoholic fermentation (Figure 2, Table I). 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) (GenBank Accession number AY130346). *H. 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 ; however, it has been shown that many non-*Saccharomyces* yeasts can survive till the end of alcoholic fermentation, especially *H. uvarum* and *I. orientalis* (Fleet and Heard, 1993 ; Fugelsang, 1997 ; Fleet, 2003 ; Zott *et al*., 2010 ; Andorra *et al*., 2011). Subden *et al.* (2003) also reported the presence of *H. uvarum* in ice-wine musts. *S. cerevisiae* (band l) only appeared after four days of alcoholic fermentation (Figure 2) and was present up to the completion of MLF (Table I). It is typical of wine fermentation that the non-*Saccharomyces* yeasts initiate 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, that controls the growth of the non-*Saccharomyces* yeasts (Fleet and Heard, 1993 ; Fleet, 2003 ; Goddard, 2008 ; Salvadó *et al*., 2011).

b. Merlot wine

PCR-based DGGE analysis using bacteria specific primers during inoculated alcoholic fermentation 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 (Table 2). The profile during spontaneous MLF (Figure 3) was the same as for day nine of the inoculated alcoholic fermentation, except for one band that disappeared during the ninth week of the MLF. The bands 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 of MLF. This band was presumptively identified as an uncultured bacterial clone DBF1G4 (98 % homology) (GenBank Accession number DQ190157). Band n was present throughout the alcoholic fermentation process and only disappeared during week nine of MLF (Figure 3). This band was identified as *E. sakazakii* (*Cronobacter* sp.) (96 % homology) (GenBank Accession number AY752939). Bands o, p and q were present throughout both fermentation processes. Band p was identified as *E. sakazakii* (*Cronobacter* sp.) (97 % homology) (GenBank Accession number AY752939) and band o was identified as *P. agglomerans* (98 % homology) (GenBank Accession number DQ530141). Band q was identified as an uncultured bacterial clone RSA1 (95 % homology) (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 bacterial clone RSA1 (95 % homology) (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

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several bands when only one species is present (Coenye and Vandamme, 2003), which can explain why there are two bands representing *E. sakazakii* (*Cronobacter* sp.) and two bands representing uncultured bacterial clone RSA1 present in the DGGE profile.

PCR-based DGGE analysis using yeast specific primers during alcoholic fermentation and MLF showed that only one band (band s) was present during both fermentation processes and that this microbe died-off during week eleven of MLF. This band was identified as *S. cerevisiae* (100 % homology) (GenBank Accession number AY601161).

CONCLUSIONS

Analysis of both Pinotage and Merlot wines during alcoholic fermentation and MLF showed that these fermentations are carried out by complex microbial populations that consist of a succession of yeast and bacterial species. *S. cerevisiae* was shown to be the principle wine yeast by PCR-DGGE but there are also many other microorganisms that may have contributed to the wine flavour profile and quality (Fleet and 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 winemaking process at the time of harvesting the grapes or from the winery environment. 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.

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