PCR and DGGE Detection Limits for Wine Spoilage Microbes

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In this study the culture-independent technique, polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE), was investigated for the early detection and identification of possible spoilage microbes in wine. PCR and DGGE conditions were successfully optimised with the universal primers HDA1[GC] and HDA2, the bacteria-specific primers WBAC1[GC]-WBAC2, and the yeast-specific primers NL1[GC] and LS2. PCR and DGGE detection limits were determined for *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Acetobacter pasteurianus* and *Brettanomyces bruxellensis* when inoculated into sterile saline solution (SSS) and white wine at 10^6 cfu/mL respectively. PCR detection limits were more sensitive (10^1 to 10^2 cfu/mL) than DGGE detection limits (10^1 to 10^4 cfu/mL), with the exception of *B. bruxellensis*, which had higher PCR and DGGE detection limits than the other reference microbes. PCR-DGGE analysis was also used successfully to detect and identify *Lb. plantarum*, *A. pasteurianus* and *B. bruxellensis* at a concentration of 10^8 cfu/mL as part of mixed populations in SSS and white wine. PCR detection limits of 10^1 cfu/mL were determined for all three reference microbes in mixed populations. The DGGE detection limits were higher for mixed populations when compared to single strains.

INTRODUCTION

Wine is the product of complex microbiological processes, with interactions between diverse species of yeasts, bacteria and mycelial fungi (Fleet, 1993; Querol & Ramón, 1996). *Saccharomyces cerevisiae* is important in winemaking, since they are responsible for alcoholic fermentation. However, yeasts can also cause spoilage during fermentation, ageing and after bottling (Fleet, 1993). The main fermenting yeast that dominates during alcoholic fermentation is *Saccharomyces cerevisiae*, and today it is mostly added as a selected pure starter culture.

It is not only yeasts that are important in winemaking, but bacteria also play an important role in wine quality (Andorrà *et al*., 2008) and are represented by two groups, namely lactic acid bacteria (LAB) and acetic acid bacteria (AAB). *Oenococcus oeni* is mainly responsible for malolactic fermentation (MLF), but other genera of LAB, such as *Lactobacillus*, *Leuconostoc* and *Pediococcus*, also play a key role in winemaking and may cause wine spoilage under specific conditions (Rankine, 1995). AAB such as *Acetobacter aceti* and *Acetobacter pasteurianus* are present in wine and may cause volatile acidity through the oxidation of ethanol to acetaldehyde and acetic acid (Fleet, 1993).

Most microbial species in wine have been identified using conventional microbiological methods (Lopez *et al*., 2003). However, these methods have limitations in the identification and classification of microbes (Muyzer, 1999), and are often time consuming and labour intensive (Hernán-Gómez *et al*., 2000; Kopke *et al*., 2000). It is often difficult to assess the true microbial diversity in an ecosystem (Giraffa & Neviani, 2001) and to cultivate all the viable microbes because of the complex conditions under which these microbes grow in their natural environment (Muyzer, 1999).

Culture-independent molecular techniques make it possible to study the total microbial DNA isolated from mixed microbial populations in order to detect, identify and characterise individual microbes in food ecosystems (Hugenholtz & Pace, 1996). Genetic fingerprinting of complex microbial populations (Muyzer, 1999) is currently used extensively to study the microbial ecology of wine fermentations (Cocolin *et al*., 2000; Mills *et al*., 2002; Renouf *et al*., 2007). Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) analysis is also used, since it allows the detection and identification of individual species, as well as the overall profiling of microbial populations (Stahl & Chapman, 1994).

The aim of this study was to evaluate three primer pairs, including universal, bacteria-specific and yeast-specific, for the identification and detection of microbes present in wine. The detection limit for each wine spoilage microbe *(Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Acetobacter pasteurianus* and *Brettanomyces bruxellensis*) in sterile saline solution (SSS) and sterile white wine was determined with the three primer pairs using PCR-based DGGE analysis. *Lactobacillus plantarum*, *A. pasteurianus* and *B. bruxellensis* were also inoculated as mixed microbial populations in order to determine the detection limits of the reference microbes when present in mixed populations.

MATERIALS AND METHODS

Microbial strains, media and growth conditions

The reference microbial species and strains selected for the study were the LAB *P. pentosaceus* LMG 1361 and *L. plantarum* LMG 13556, the AAB *A. pasteurianus* DSM 3509[T], and the yeast *B. bruxellensis* ISA 1649. The microbes and their specific culturing requirements are summarised in Table 1. The standard growth

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curves of each reference microbe were used to determine the inoculum size.

Inoculation with single strains

Sauvignon blanc white wine, produced at the Department of Viticulture and Oenology, Stellenbosch University during the 2008 season and sterilised with Velcorin (Sigma-Aldrich), and SSS were inoculated with 10^6 cfu/mL of a specific wine microbial strain (*L. plantarum*, *P. pentosaceus*, *A. pasteurianus* or *B. bruxellensis*) to determine PCR-based DGGE detection limits for the universal, bacteria-specific and yeast-specific primers. The wine was sterilised with Velcorin (Sigma-Aldrich) 48 h before inoculation of the reference microbes. Velcorin was prepared and diluted in a 1:4 ratio with 100% ethanol (Merck), and 200 µL/L of the dilution was added to the wine.

A 100 mL sample of both sterile white wine and SSS was inoculated with a single microbial strain at a concentration of 10^6 cfu/mL. A dilution series of the inoculated SSS and sterile white wine was made in SSS, and each dilution was pour plated (in duplicate) on the appropriate cell count agar (Table 2), after which the plates were incubated and the colonies were counted. Sterile white wine and SSS served as controls and no growth was detected after incubation. PCR and DGGE detection limits were also determined for each dilution. All experiments were done in triplicate.

Inoculation with mixed strains

Lactobacillus plantarum was selected as a representative wine LAB, *A. pasteurianus* as a representative wine AAB, and *B. bruxellensis* as the representative wine yeast. SSS and sterile

TABLE 1

Culturing media, incubation times and temperatures used for the reference microbes in SSS and sterile white wine.

Microbe	Culturing media	pH[a]	Incubation time (h)	Incubation temperature (°C)
Pediococcus pentosaceus	MRS[b] supplemented with 40 g/L D(-) fructose (Merck), 20 g/L D(+) glucose (Merck), 4 g/L L(-) malic acid (Merck), 1 g/L Tween 80 (Merck) and 4% (v/v) ethanol	4.6	48	30
Lactobacillus plantarum	MRS supplemented with 40 g/L D(-) fructose (Merck), 20 g/L D(+) glucose (Merck), 4 g/L L(-) malic acid (Merck), 1 g/L Tween 80 (Merck) and 4% (v/v) ethanol	4.6	48	30
Acetobacter pasteurianus	MRS supplemented with 2% (v/v) ethanol	5.5	72	30
Brettanomyces bruxellensis	YPD[c] supplemented with 6% (v/v) ethanol	6.5	96	25

[a]pH adjusted with 1 M HCl according to The South African Wine Laboratories Association (2002).
[b]MRS = de Man, Rogosa and Sharpe broth (Biolab).
[c]YPD = yeast peptone dextrose broth (Biolab).

TABLE 2

Cell count agar, incubation times and temperatures used for the reference microbes inoculated as mixed cultures in SSS and sterile white wine.

Microbe	Agar	pH[a]	Incubation time (d)	Incubation temperature (°C)	Antibiotics (mg/L)
Lactobacillus plantarum	MRS[b]	6.5	5-7	30	Actistab; 100 Kanamycin sulphate; 25
Acetobacter pasteurianus	MRS supplemented with 2% (v/v) ethanol	5.5	5-7	30	Actistab; 100 Streptomycin sulphate; 25
Brettanomyces bruxellensis	DBDM[c] agar	5.4	11-14	25	Streptomycin sulphate; 25 Kanamycin sulphate; 25

[a]pH adjusted with 1 M HCl according to The South African Wine Laboratories Association (2002).
[b]MRS = de Man, Rogosa and Sharpe broth (Biolab).
[c]DBDM = *Dekkera*/*Brettanomyces* differential medium (6.7 g/L yeast nitrogen base YNB (Difco), 100 mg/L p-coumaric acid (Sigma-Aldrich), 22 mg/L bromocresol green (Merck), 6% (v/v) ethanol and 20 g/L bacteriological agar (Biolab)) (Rodrigues *et al*., 2001).

white wine were inoculated with mixed microbial populations containing 10^8 cfu/mL of each of the representative strains in order to determine PCR-DGGE detection limits for the universal and bacteria-specific primers with the mixtures of microbes. The standard growth curves of each reference microbe were used to determine the inoculum size. The following combinations of *L. plantarum*, *A. pasteurianus* and *B. bruxellensis* were inoculated into SSS and sterile white wine: *L. plantarum*, *A. pasteurianus* and *B. bruxellensis*; *L. plantarum* and *B. bruxellensis*; *L. plantarum* and *A. pasteurianus*; or *B. bruxellensis* and *A. pasteurianus*.

A dilution series of the inoculated SSS and sterile white wine was made in SSS, and each dilution was pour plated (in duplicate) on the appropriate cell count agar, after which the plates were incubated and the colonies were enumerated (Table 2). The cell count agar was supplemented with specific antibiotics in order to eliminate the growth of unwanted yeasts, AAB and LAB. Sterile white wine and SSS served as controls and no growth was detected after incubation. PCR and DGGE detection limits were also determined for each dilution. All experiments were done in triplicate.

DNA isolation

DNA was extracted from the inoculated microbes in the SSS, as well as from the white wine. DNA isolation was carried out according to the modified method of Wang and Levin (2006). Two mL of the inoculated medium was centrifuged for 10 min at 6 000 x *g*, after which the supernatant was discarded. The pellet was re-suspended in 250 μL of SSS, and 250 μL of the suspension was mixed with 250 μL double-strength TZ (2 x TZ), consisting of 4% (v/v) Triton X-100 (Merck) and 5 mg/mL sodium azide (Merck) in 0.1 M Tris-HCl (Fluka); pH 8.0. The sample tubes were boiled for 10 min in a water bath to lyse the cells, after which the microcentrifuge tubes were placed on ice for 5 min. The microcentrifuge tubes were then centrifuged for 5 min at 10 000 x *g* and 200 μL of the supernatant was extracted and purified using a Micropure-EZ column (Millipore). All experiments were done in triplicate.

PCR

The V3 variable region of the 16S ribosomal RNA (rRNA) gene for the bacterial reference strains, and the D1/D2 region of the 26S rRNA gene for the yeast reference strain were amplified using the universal primers HDA1[GC] (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G ACT CCT ACG GGA GGC AGC AGT-3') and HDA2 (5'- GTA TTA CCG CGG CTG CTG GCA C-3') (250 bp fragment) (Lopez *et al*., 2003). To facilitate DGGE separation, a GC-rich sequence (GC clamp sequence is underlined) was attached to the forward primer. The PCR reactions were performed in a total volume of 40 μL containing 1 x PCR buffer, 2.5 mM MgCl$_2$, 0.8 mM dNTPs, 0.5 μM of each primer, 1.5 U *Taq* DNA polymerase (Super-Therm, Southern Cross Biotechnology, Cape Town, South Africa) and 2 μL of DNA template. Thermal cycling was carried out with a thermal cycler (Eppendorf Mastercycler Personal, Merck, Hamburg, Germany) with an initial denaturation at 94˚C for 4 min, followed by 30 cycles of denaturation at 94˚C for 30 s, annealing at 56˚C for 30 s and elongation at 68˚C for 60 s. A final elongation at 68˚C for 7 min was also performed.

The V7 to V8 variable region of the 16S rRNA gene for the bacterial reference strains was amplified using the bacteria-specific primers WBAC1[GC] (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCC CCC GGT CGT CAG CTC GTG TCG TGA GA-3') and WBAC2 (5'-CCC GGG AAC GTA TTC ACC GCG-3') (GC clamp sequence is underlined) (320 bp fragment), which are based on the primers described by Lopez *et al*. (2003). According to Lopez *et al*. (2003), no specific primers have been reported for AAB and the WBAC1[GC]-WBAC2 primers could successfully be used for the amplification of both LAB and AAB associated with wine. The PCR reactions were performed in a total volume of 50 μL, containing 1 x PCR buffer, 1.5 mM MgCl$_2$, 0.8 mM dNTPs, 0.2 μM of each primer, 2.5 U *Taq* DNA polymerase (Super-Therm) and 3 μL of DNA template. Thermal cycling was carried out with an initial denaturation at 95˚C for 5 min, followed by 30 cycles of denaturation at 95˚C for 60 s, annealing at 57˚C for 30 s and elongation at 72˚C for 60 s. A final elongation at 72˚C for 5 min was also performed during the reaction.

The D1/D2 region of the 26S rRNA gene of the yeast reference strain 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') and LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (GC clamp sequence is underlined) (250 bp fragment) (O'Donnell, 1993). The PCR reactions were performed in a total volume of 25 μL containing 1 x PCR buffer, 3 mM MgCl$_2$, 0.4 mM dNTPs, 0.6 μM of each primer, 1.25 U *Taq* DNA polymerase (Super-Therm), 1 μL 99% (v/v) dimethyl sulphoxide (DMSO) (Merck) and 1 μL of DNA template. The PCR reaction consisted of an initial 5 min denaturation at 95˚C, followed by 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. The reaction was completed with a final elongation at 72˚C for 7 min.

The PCR amplicons, together with a positive and negative control, were separated on a 1.5% (m/v) agarose gel (stained with 0.02 μL/mL ethidium bromide) in 0.5 x TBE electrophoresis buffer. The PCR fragments were visualised under an ultraviolet transilluminator (Vilber Lourmat, Marne-La-Vallée, France).

DGGE analysis

The PCR fragments obtained from the amplification using the HDA1[GC]–HDA2, WBAC1[GC]–WBAC2 and NL1[GC]–LS2 primers were resolved using DGGE analysis, performed with the BioRad DCode Universal Mutation Detection System (Bio-Rad Laboratories, Cape Town, South Africa). PCR products were applied directly onto 8% (m/v) polyacrylamide gels with a denaturing gradient of between 45% and 70% of 7 M urea (Merck) and 40% (v/v) formamide (Merck) in a 1 x TAE buffer. The electrophoresis was performed at a constant voltage of 130 mV for 5 h and a constant temperature of 60˚C. The gels were stained in 1 x TAE buffer containing ethidium bromide (94 μl/L), and the fragments were visualised under an ultraviolet transilluminator (Vilber Lourmat).

RESULTS AND DISCUSSION

Optimisation of PCR-DGGE

The universal primers, HDA1[GC]–HDA2, the bacteria-specific primers, WBAC1[GC]-WBAC2, as well as the yeast-specific primers NL1[GC]–LS2, were selected for the amplification of DNA isolated from the reference microbes *P. pentosaceus*, *L. plantarum*, *A. pasteurianus* and *B. bruxellensis*. The primers successfully amplified the specific yeast and bacterial species evaluated in this study.

Amplicons obtained after PCR amplification were successfully resolved using DGGE analysis. Approximately 250 bp amplicons, amplified with the HDA1[GC]–HDA2 primers, were successfully resolved using DGGE analysis. *Pediococcus pentosaceus* and *B. bruxellensis* showed the same migration distances in the DGGE gel. This means that it would not be possible to distinguish these two microbial species from each other on a DGGE gel when amplified using these universal primers.

The 320 bp amplicons, amplified with the WBAC1[GC]-WBAC2 primers, were found to have different migration distances in the DGGE gel, and it would thus be possible to distinguish between these bacterial strains when separated on a DGGE polyacrylamide gel. The reference bacteria would also have different positions in a reference ladder, which could be used for species identification.

The optimised DGGE conditions could be used for reference ladders, as an alternative to the sequencing of DGGE bands, to presumptively identify the microbial species (Ercolini, 2004) inoculated into SSS and sterile white wine. The identification of the microbial species is achieved by comparing the PCR fragment migration distances in the DGGE polyacrylamide gels with those of the reference species present (Ercolini, 2004).

Detection limits for single microbes

The performance of PCR-DGGE analysis for the detection and identification of wine spoilage yeasts and bacteria was evaluated and the results were confirmed with pour plating for enumeration. After PCR and DGGE optimisation, the limit of microbial detection by PCR-DGGE analysis was determined for *P. pentosaceus*, *L. plantarum*, *A. pasteurianus* and *B. bruxellensis* when each microbial strain was separately inoculated into SSS and sterile white wine at 10^6 cfu/mL.

Acetobacter pasteurianus

The PCR and DGGE detection limits obtained for *A. pasteurianus* are summarised in Table 3. PCR amplicons were successfully obtained for the dilution samples when *A. pasteurianus* was inoculated into SSS and white wine and amplified with the HDA1[GC]–HDA2 and WBAC1[GC]-WBAC2 primer pairs. The PCR detection limits were determined as 10^1 cfu/mL in both SSS and white wine.

The PCR fragments were successfully resolved using DGGE analysis, and DGGE detection limits of 10^2 cfu/mL were determined for *A. pasteurianus* when inoculated into SSS for both primer pairs. When the inoculation was done in white wine and the fragments amplified with the WBAC1[GC]-WBAC2 primers, a slightly higher DGGE detection limit of 10^3 cfu/mL was determined than when the inoculation was done in SSS (10^2 cfu/mL). As was the case for SSS, the DGGE detection limit for *A. pasteurianus* in white wine, and with the HDA1[GC]–HDA2 primers, was 10^2 cfu/mL.

Lactobacillus plantarum

When *L. plantarum* was inoculated into SSS and white wine, PCR detection limits of 10^1 cfu/mL were determined when amplified with the HDA1[GC]–HDA2 primers (Table 3). When amplified with the bacteria-specific primers (WBAC1[GC]-WBAC2), a PCR

TABLE 3

PCR and DGGE detection limits for reference microbial strains inoculated singly (10^6 cfu/mL).

Microbe	Inoculation medium	Primers	PCR detection limit (cfu/mL)	DGGE detection limit (cfu/mL)
A. pasteurianus	SSS	HDA1[GC]/HDA2	10^1	10^2
		WBAC1[GC]/WBAC2	10^1	10^2
	White wine	HDA1[GC]/HDA2	10^1	10^2
		WBAC1[GC]/WBAC2	10^1	10^3
L. plantarum	SSS	HDA1[GC]/HDA2	10^1	10^1
		WBAC1[GC]/WBAC2	10^1	10^2
	White wine	HDA1[GC]/HDA2	10^1	10^2
		WBAC1[GC]/WBAC2	10^2	10^3
P. pentosaceus	SSS	HDA1[GC]/HDA2	10^1	10^1
		WBAC1[GC]/WBAC2	10^1	10^2
	White wine	HDA1[GC]/HDA2	10^2	10^2
		WBAC1[GC]/WBAC2	10^2	10^4
B. bruxellensis	SSS	HDA1[GC]/HDA2	10^1	10^4
		NL1[GC]/LS2	10^4	10^5
	White wine	HDA1[GC]/HDA2	10^3	10^3
		NL1[GC]/LS2	10^4	10^5

detection limit of 10^1 cfu/mL was determined in SSS, with a slightly higher PCR detection limit of 10^2 cfu/mL when white wine was used as the inoculation medium.

The PCR fragments were successfully resolved with DGGE analysis, and a DGGE detection limit of 10^1 cfu/mL was determined when *L. plantarum* was inoculated into SSS and amplified with the universal primers (HDA1^{GC}–HDA2) (Fig. 1). A DGGE detection limit of 10^2 cfu/mL was found when the amplifications was done with the WBAC1^{GC}-WBAC2 primers (Table 3). The DGGE limits in white wine were 10^2 cfu/mL and 10^3 cfu/mL for the universal and bacteria-specific primers respectively (Table 3).

Pediococcus pentosaceus

With the inoculation of *P. pentosaceus* into SSS, PCR detection limits of 10^1 cfu/mL were determined for both the universal and the bacteria-specific primer pairs (Table 3). A slightly higher PCR detection limit of 10^2 cfu/mL was determined for the HDA1^{GC}–HDA2, as well as for the WBAC1^{GC}-WBAC2 primer pairs, with inoculation of *P. pentosaceus* into white wine.

The PCR fragments were resolved using DGGE analysis, and when *P. pentosaceus* was inoculated into SSS a DGGE detection limit of 10^1 cfu/mL was determined for the PCR fragments amplified with the HDA1^{GC}–HDA2 primers. A DGGE detection limit of 10^2 cfu/mL was determined for *P. pentosaceus* inoculated into SSS when amplified with the WBAC1^{GC}-WBAC2 primers. The DGGE detection limit for *P. pentosaceus* in white wine was determined as 10^2 cfu/mL when amplified with the universal primer pair (Table 3). When the fragments that had been amplified with the WBAC1^{GC}-WBAC2 primers were resolved with DGGE, a detection limit of 10^4 cfu/mL was determined. It was found that it was possible to detect lower cell concentrations of *P. pentosaceus* with the HDA1^{GC}–HDA2 primer pair in both the SSS and the white wine than with the WBAC1^{GC}-WBAC2 primer pair.

Brettanomyces bruxellensis

PCR detection limits of 10^1 cfu/mL and 10^3 cfu/mL were determined when *B. bruxellensis* was amplified with the universal primers in SSS and white wine respectively (Table 3). When amplified with the yeast-specific primers NL1^{GC}–LS2, a PCR detection limit of 10^4 cfu/mL^{-1} was determined when *B. bruxellensis* was inoculated into both SSS and white wine. The PCR detection limits were higher than expected for the yeast *B. bruxellensis*, and generally higher than the detection limits determined for the reference wine bacteria.

A DGGE detection limit of 10^4 cfu/mL was determined for SSS with the universal primers (HDA1^{GC}–HDA2), and a DGGE detection limit of 10^5 cfu/mL was observed when amplified with the yeast-specific primers (NL1^{GC}–LS2) (Table 3). When *B. bruxellensis* was inoculated into white wine, a DGGE detection limit of 10^3 cfu/mL was obtained with the universal primers. The NL1^{GC}–LS2 primers could only resolve concentrations of *B. bruxellensis* of greater than 10^5 cfu/mL in white wine and SSS with DGGE (Table 3), and did not give reproducible and reliable results for the determination of PCR and DGGE detection limits in white wine. This could possibly mean that the yeast-specific primer pair is less sensitive than the universal primers, thus more DNA is required for PCR amplification with the NL1^{GC}–LS2 primer pair. This would suggest that NL1^{GC}–LS2 would not be a suitable primer pair for the detection of *B. bruxellensis* using PCR-based DGGE analysis.

The results obtained from the determination of PCR and DGGE detection limits, when 10^6 cfu/mL of the reference microbial strains were separately inoculated into SSS and white wine, illustrated that the universal, bacteria-specific and yeast-specific primers used in this study could be used successfully to detect and identify spoilage microbes that are associated with winemaking. When the inoculations were done in white wine, higher detection limits were obtained than when the inoculations were done in
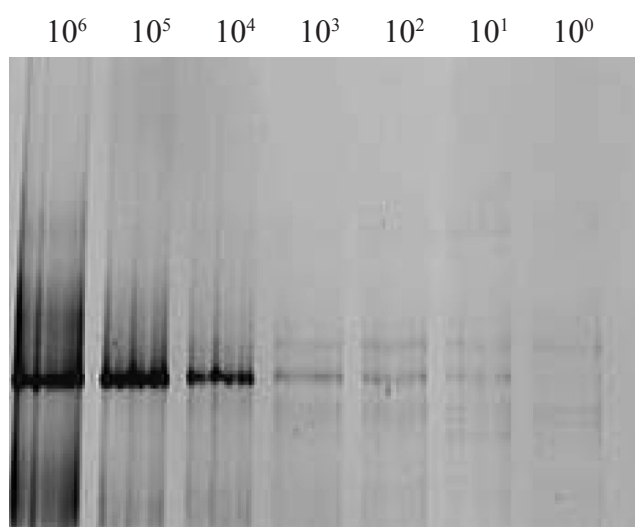
$$10^6 \quad 10^5 \quad 10^4 \quad 10^3 \quad 10^2 \quad 10^1 \quad 10^0$$

FIGURE 1

DGGE analysis of the different concentrations of *L. plantarum* (10^6–10^0 cfu/mL) inoculated into sterile saline solution and amplified with the primers HDA1^{GC} and HDA2.

SSS. This may be due to the presence of inhibitors that are present in wine. Plant materials, such as polysaccharides, plant lipids and polyphenols are known to inhibit PCR reactions, which ultimately will also influence the outcome of the DGGE detection limit results (Lodhi *et al.*, 1994). The plant material and inhibitory substances that were extracted during DNA isolation could also have had an influence on the PCR amplification of the DNA template, and could cause a decrease in the sensitivity of this detection method (Prakitchaiwattana *et al.*, 2004). The sensitivity of the primers used in this study differed in terms of PCR and DGGE detection limits. PCR was found to be more sensitive than DGGE, as the PCR detection limits were found to be lower for most samples. The universal primers (HDA1^{GC}–HDA2) and the bacteria-specific primers (WBAC1^{GC}-WBAC2) had similar sensitivity for the PCR amplification of the DNA templates from the inoculated samples.

Detection limits for mixed microbes

Several bacterial and yeast species are present in wine during alcoholic fermentation and MLF (Prakitchaiwattana *et al.*, 2004). Detection limits of the reference microbes inoculated into SSS and white wine therefore were determined as part of a mixed population with the universal and bacteria-specific primers. Due to the high detection limits obtained with the NL1^{GC}–LS2 primers, it was decided not to use this primer pair for the detection of *B. bruxellensis* in mixed microbial populations.

The performance of PCR-based DGGE analysis was thus evaluated in detecting individual wine microbial strains in cell suspensions containing a variety of microbial populations.

Acetobacter pasteurianus and *Lactobacillus plantarum*

When *A. pasteurianus* and *L. plantarum* were inoculated into SSS and white wine, a PCR detection limit of 10^1 cfu/mL was obtained for both bacterial species when amplified with the HDA1^{GC}–HDA2 and WBAC1^{GC}-WBAC2 primers (Table 4). The

PCR detection limits determined for these two bacteria compared well with the results for the detection limits of the single reference microbial strains inoculated into SSS and white wine. The PCR detection limits were observed as 10^1 cfu/mL for both bacterial species, except for *L. plantarum*, which had a detection limit of 10^2 cfu/mL when inoculated as a single strain in white wine and amplified with the WBAC1^{GC}-WBAC2 primer pair (Table 3).

The DGGE detection limits in SSS were determined to be 10^3 cfu/mL for both *A. pasteurianus* and *L. plantarum* when amplified with the universal primers, and 10^1 cfu/mL when amplified with the bacteria-specific primers. When *A. pasteurianus* and *L. plantarum* were inoculated into white wine, a DGGE detection limit of 10^1 cfu/mL was determined for *A. pasteurianus*, and a higher detection limit of 10^4 cfu/mL was found for *L. plantarum* when amplified with the HDA1^{GC}–HDA2 primers (Table 4). When the PCR amplicons that were amplified with the WBAC1^{GC}-WBAC2 primers were resolved using DGGE, a detection limit of 10^1 cfu/ mL was determined for both *A. pasteurianus* and *L. plantarum* (Table 4). This primer pair was capable of amplifying a smaller amount of cells and was thus more sensitive than the HDA1^{GC}– HDA2 primers in amplifying a mixed population of these wine bacteria. When compared to the DGGE detection limits of the reference microbes inoculated as single strains, it was observed that the HDA1^{GC}–HDA2 primer pair was more sensitive than the WBAC1^{GC}-WBAC2 primer pair in the detection of the single microbial strains, but as part of mixed populations it was observed that the WBAC1^{GC}-WBAC2 primer pair was more sensitive.

Acetobacter pasteurianus and *Brettanomyces bruxellensis*

When the wine AAB, *A. pasteurianus*, and the wine yeast, *B. bruxellensis*, were inoculated into SSS and white wine, a PCR detection limit of 10^1 cfu/mL was determined for both these microbes when amplified with both the universal and bacteria-specific primers (Table 4). As was expected, the bacteria-specific
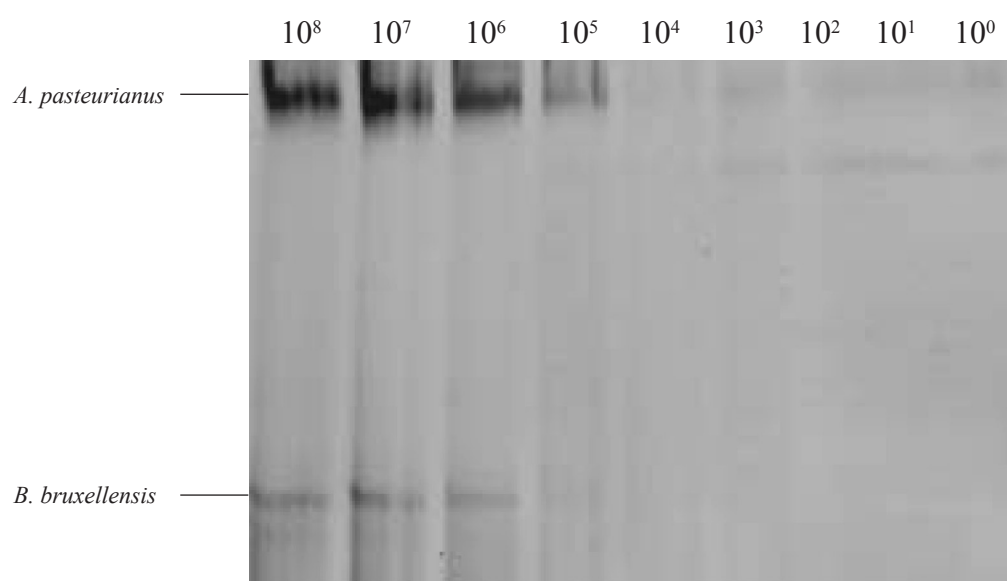

FIGURE 2

DGGE analysis of the different concentrations of *A. pasteurianus* and *B. bruxellensis* (10^8 to 10^0 cfu/mL) inoculated into sterile white wine as a mixture and amplified with the primers HDA1^{GC} and HDA2.

TABLE 4

PCR and DGGE detection limits for the inoculation of sterile saline solution and sterile white wine with mixed populations (10^8 cfu/mL of each strain).

Microbe	Medium	Primers	PCR detection limit (cfu/mL)				DGGE detection limit (cfu/mL)			
			1[a]	2[b]	3[c]	4[d]	1	2	3	4
A. pasteurianus	SSS	HDA1^{GC}/HDA2	10^1	10^1	NI[e]	10^1	10^3	10^1	NI	10^1
		WBAC1^{GC}/WBAC2	10^1	10^1	NI	10^1	10^1	10^5	NI	10^1
	White wine	HDA1^{GC}/HDA2	10^1	10^1	NI	10^1	10^1	10^5	NI	10^7
		WBAC1^{GC}/WBAC2	10^1	10^1	NI	10^1	10^1	10^5	NI	10^2
L. plantarum	SSS	HDA1^{GC}/HDA2`	10^1	NI	10^1	10^1	10^3	NI	10^1	10^1
		WBAC1^{GC}/WBAC2	10^1	NI	10^1	10^1	10^1	NI	10^4	10^1
	White wine	HDA1^{GC}/HDA2	10^1	NI	10^1	10^1	10^4	NI	10^1	10^1
		WBAC1^{GC}/WBAC2	10^1	NI	10^1	10^1	10^1	NI	10^5	10^2
B. bruxellensis	SSS	HDA1^{GC}/HDA2	NI	10^1	10^1	10^1	NI	10^1	10^1	10^1
	White wine	HDA1^{GC}/HDA2	NI	10^1	10^1	10^1	NI	10^6	10^1	10^1

[a]1 = combination of *A. pasteurianus* and *Lb. plantarum*.
[b]2 = combination of *A. pasteurianus* and *B. bruxellensis*.
[c]3 = combination of *Lb. plantarum* and *B. bruxellensis*.
[d]4 = combination of *A. pasteurianus*, *Lb. plantarum* and *B. bruxellensis*.
[e]NI = not inoculated.

primers could not amplify the yeast DNA from *B. bruxellensis*. When compared to the PCR detection limits of the single reference microbial strains, it was observed that the same PCR detection limit was determined using the universal primers (HDA1^{GC}–HDA2), but that a higher detection limit of 10^3 cfu/mL was observed for *B. bruxellensis* inoculated into white wine when amplified with the HDA1^{GC}–HDA2 primers.

The PCR amplicons were successfully resolved on a DGGE gel and a DGGE detection limit of 10^1 cfu/mL was determined for *A. pasteurianus* and *B. bruxellensis* when inoculated into SSS and when amplified with the universal primers. When amplified with the WBAC1^{GC}-WBAC2 primers, a DGGE detection limit of 10^5 cfu/mL was obtained for *A. pasteurianus* (Table 4). When the inoculation was done in white wine, DGGE detection limits of 10^5 cfu/mL and 10^6 cfu/mL was determined for *A. pasteurianus* and *B. bruxellensis* respectively, using HDA1^{GC}–HDA2 (Table 4, Fig. 2). A DGGE detection limit of 10^5 cfu/mL was also observed for *A. pasteurianus* in white wine when amplified with the bacteria-specific primers (Table 4).

The two primer pairs had a similar sensitivity for the amplification of this wine bacterium (*A. pasteurianus*) when simultaneously inoculated with a wine yeast. When compared to the results obtained from the inoculation of the single microbial reference strains (Table 3), it was observed that lower detection limits were obtained for the inoculation of the single microbial strains than when inoculated as part of a mixed microbial population. When inoculated as a mixture at a concentration of 10^8 cfu/mL, *B. bruxellensis* and *A. pasteurianus* had lower DGGE detection limits, of 10^1 cfu/mL, than when either of these two strains was inoculated as a single strain in SSS (10^2 cfu/mL and 10^4 cfu/mL for *A. pasteurianus* and *B. bruxellensis* respectively). The reason

for these findings is uncertain, and no similar studies on these microbes have been reported in the literature. Therefore, further research is needed to explain why a lower detection limit was observed for these microbial species in a mixed population than as single microbial strains.

Lactobacillus plantarum and *Brettanomyces bruxellensis*

When *L. plantarum* and *B. bruxellensis* were inoculated into SSS and white wine and amplified with the HDA1^{GC}–HDA2 primers, as well as WBAC1^{GC}-WBAC2 , PCR detection limits of 10^1 cfu/mL were determined for both the wine bacterium and the yeast (Table 4). When *L. plantarum* and *B. bruxellensis* were inoculated into SSS and amplified with HDA1^{GC}–HDA2, a DGGE detection limit of 10^1 cfu/mL was determined for both these microbes. When amplified with the WBAC1^{GC}-WBAC2 primers a higher DGGE detection limit, of 10^4 cfu/mL, was determined for *L. plantarum* when compared to amplification with the universal primer pair (Table 4). When *L. plantarum* and *B. bruxellensis* were inoculated into white wine, a DGGE detection limit of 10^1 cfu/mL was determined for both the microbial species. PCR amplicons that were amplified with the WBAC1^{GC}-WBAC2 primers were resolved with DGGE analysis and gave a detection limit of 10^5 cfu/mL for *L. plantarum*.

When compared to the results obtained for the inoculation of the single microbial strains (Table 3), it was observed that higher DGGE detection limits, of 10^4 cfu/mL and 10^3 cfu/mL, were obtained for *B. bruxellensis* inoculated as a single microbial strain and when amplified with HDA1^{GC}–HDA2 in SSS and white wine respectively. Regarding the DGGE detection limits, it was also observed that the WBAC1^{GC}-WBAC2 primer pair was more sensitive when *L. plantarum* was inoculated as a single strain

than when inoculated with *B. bruxellensis*. The results with the HDA1^GC–HDA2 primer pair compared well when *L. plantarum* was inoculated as a single strain and when it was inoculated as part of a mixed population. A DGGE detection limit of 10^1 cfu/mL was obtained for *L. plantarum* when inoculated as a single microbial strain and as part of a mixed microbial population, and DGGE detection limits of 10^1 cfu/mL and 10^2 cfu/mL were determined when inoculated as a single microbial strain and as part of a mixed population respectively.

Acetobacter pasteurianus, Lactobacillus plantarum and *Brettanomyces bruxellensis*

The PCR and DGGE detection limits determined for the inoculation of the mixed population of *A. pasteurianus*, *L. plantarum* and *B. bruxellensis* into SSS and white wine are given in Table 4. The PCR detection limit for all three of the microbes was determined as 10^1 cfu/mL when inoculated into SSS and white wine and when amplified with both the HDA1^GC–HDA2 and WBAC1^GC-WBAC2 primer pairs. Since the bacteria-specific primer pair WBAC1^GC-WBAC2 is not specific for the amplification of yeast species, it was not expected that this primer pair would amplify *B. bruxellensis*, and thus there is no detection limit for *B. bruxellensis* when amplified with WBAC1^GC-WBAC2 .

The DGGE detection limits for the reference microbes inoculated into SSS as part of a mixed population were determined at 10^1 cfu/mL when amplified with both primer pairs (Table 4). When inoculated into white wine and amplified with the HDA1^GC–HDA2 primers, a DGGE detection limit of 10^1 cfu/mL was determined for *L. plantarum* and *B. bruxellensis*, while a DGGE detection limit of 10^7 cfu/mL was determined for *A. pasteurianus*. When the two bacterial species and the yeast species were inoculated into white wine and amplified with the WBAC1^GC-WBAC2 primers, a DGGE detection limit of 10^2 cfu/mL was determined for both the bacterial species. The results indicate that the WBAC1^GC-WBAC2 primer pair was more sensitive and specific in the amplification of the wine bacteria than the HDA1^GC–HDA2 primers, and that it could detect lower concentrations of the bacterial species.

When compared to the inoculation of single microbial strains in SSS and white wine (Table 3), it was observed that the DGGE detection limits were lower, at 10^1 cfu/mL for the three microbial species, than when inoculated as single strains in SSS. It was also observed that the DGGE detection limits were lower for the reference microbial strains when inoculated as part of a mixed population into white wine, with the exception of *A. pasteurianus*, which had a DGGE detection limit of 10^7 cfu/mL when inoculated in white wine as a single microbial strain.

CONCLUSIONS

The results obtained in this study indicate that PCR-DGGE analysis can successfully be used for the detection of the potential wine spoilage microbes *P. pentosaceus*, *L. plantarum*, *A. pasteurianus* and *B. bruxellensis*, both as single microbes and as part of mixed populations inoculated into SSS and white wine. However, it is extremely important to optimise the PCR and DGGE conditions that will be used in the assay. PCR and DGGE conditions were successfully optimised for *P. pentosaceus*, *L. plantarum*, *A. pasteurianus* and *B. bruxellensis* with a universal, a bacteria-specific and a yeast-specific primer pair. These optimised DGGE profiles can be used presumptively to identify microbial species

present in wine samples by constructing a reference ladder. It was found that the yeast-specific primer pair could not amplify low concentrations of microbial cells.

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