Selection and Characterisation of *Oenococcus oeni* and *Lactobacillus plantarum* South African Wine Isolates for Use as Malolactic Fermentation Starter Cultures

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Submitted for publication: May 2011
Accepted for publication: September 2011

Key words: Malolactic fermentation, *Oenococcus oeni*, *Lactobacillus plantarum*, starter cultures, enzyme encoding genes

This study focused on characterising 23 *Oenococcus oeni* and 19 *Lactobacillus plantarum* strains isolated from the South African wine environment for the development of potential commercial malolactic fermentation (MLF) starter cultures. These strains were characterised with regards to oenological important characteristics, including the genetic screening for enzyme-encoding genes (enzymes that are involved/implicated in wine aroma modification, as well as enzymes pertaining to the wholesomeness of the final wine product), their fermentation capabilities, the ability to maintain viability during MLF, as well as the volatile acidity production. A total of three *O. oeni* and three *L. plantarum* strains were selected at the completion of this study. These six strains showed the most potential during the characterisation stages of the study and were able to successfully complete MLF in Pinotage wine. It was also found that *L. plantarum* strains displayed a more diverse enzyme profile than *O. oeni* strains, particularly with regards to the presence of the aroma-modifying enzymes *β*-glucosidase and phenolic acid decarboxylase (PAD), which implies the future use of this species in the modification of the wine aroma profile and use as commercial starter culture.

INTRODUCTION

Malolactic fermentation (MLF) in wine is a deacidification process consisting of the decarboxylation of L-malic acid by the malolactic enzyme and is a result of the metabolic activity of lactic acid bacteria (LAB). During this fermentation process L-malic acid is decarboxylated to produce L-lactic acid and carbon dioxide. The three main reasons for allowing MLF to take place is the resulting deacidification, microbiological stability and the associated modifications in the aroma profile of the wine (Wibowo *et al*., 1985; Lerm *et al*., 2010). The indigenous LAB population associated with the wine environment usually belongs to the genera of *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Lonvaud-Funel, 1999). *Oenococcus oeni* is the species of LAB that is most often responsible for MLF and has shown to be able to successfully survive the challenging wine environment. Some of these challenges include high alcohol concentrations, low pH, extreme fermentation temperatures and the presence of sulphur dioxide (SO_2) (Davis *et al*., 1988; Drici-Cachon *et al*., 1996). Currently, *O. oeni* is the major LAB used in commercial starter cultures for MLF. Some *Lactobacillus* species have also displayed the ability to survive the harsh wine conditions (G-Alegría *et al*., 2004; Pozo-Bayón *et al*., 2005), of which the species *Lactobacillus plantarum* has shown the most potential as a starter culture (for review see Du Toit *et al*., 2010) and a commercial culture has recently been released by Lallemand as *Lactobacillus plantarum* V22® (Bou & Krieger, 2004; Fumi *et al*., 2010). This species of *Lactobacillus* also shows a more diverse enzymatic profile than *O. oeni* (Matthews *et al*., 2004; Spano *et al*., 2005; Mtshali *et al*., 2010), which could play an important role in the modification of the wine aroma profile (Guerzoni *et al*., 1995; Swiegers *et al*., 2005; Matthews *et al*., 2006).

There are various important criteria to consider when selecting LAB for possible use in a starter culture. These include the ability to tolerate low pH, high ethanol and SO_2 concentrations, good growth characteristics under winemaking conditions, compatibility with the selected yeast strain, the inability to produce biogenic amines and the lack of off-flavour or off-odour production (Wibowo *et al*., 1985; Britz & Tracey, 1990; Henick-Kling, 1993; Lonvaud-Funel, 2001; Ruiz *et al*., 2010; Solieri *et al*., 2010).

There are various enzymes originating from LAB that could potentially contribute to the sensory profile and wholesomeness of wines undergoing MLF. Some of the aroma-related enzymes include *β*-glucosidase, phenolic

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Aknowledgements: This study was financially supported by Anchor Yeast and THRIP.

acid decarboxylase (PAD), citrate lyase, esterase, protease, peptidases, α-acetolactate synthase and α-acetolactate reductase (Spano *et al*., 2005; Sumby *et al*., 2009; Brod *et al*., 2010; Mtshali *et al*., 2010), as well as enzymes that play a role in the production of volatile sulphur compounds, including *S*-adenosylmethionine synthase, cystathionine β/γ-lyase and glutathione reductase (Knoll *et al*., 2010). Other enzymes of interest that play a negative role in the wholesomeness of the wine, besides those enzymes implicated in biogenic amine production, include those that are involved in ethyl carbamate formation, a potential carcinogen. These enzymes are carbamate kinase, ornithine transcarbamylase and arginine deiminase, related to arginine metabolism (Liu *et al*., 1995; Arena *et al*., 2002).

The overall aim of the study was to evaluate *O. oeni* and *L. plantarum* strains isolated from South African wines for use as possible MLF starter cultures. The first objective was to characterise the isolates in order to identify potential starter strains. This was done by assessing the malic acid degradation rate of the isolates in a synthetic wine medium and screening the isolates for the presence of genes that play a role in the production of biogenic amines and ethyl carbamate, as well as other enzymes important in aroma production. The second objective was to select and evaluate the viability, MLF rate and volatile acidity production of the most promising strains in Pinotage wine inoculated after alcoholic fermentation (AF).

MATERIALS AND METHODS

Bacterial isolates, media and culture conditions

Oenococcus oeni and *L. plantarum* strains selected for use in this study were taken from the Institute for Wine Biotechnology (IWBT) (Stellenbosch University, South Africa) culture collection, as well as isolates from spontaneous MLF in Pinotage wine from the Paarl region, South Africa. Table 1 lists the bacterial strains used in this study. The identity of these strains was confirmed as *O. oeni* or *L. plantarum* (Lerm, 2010).

Representative sampling of the grape must were done for microbiological enumeration before MLF and on a weekly basis for the duration of the fermentation. The microbiological population was monitored by plate counts of colonies (CFU/mL) formed on selected media. *Lactobacillus plantarum* strains were cultivated on De Man, Rogosa and Sharpe (MRS) agar plates containing MRS broth (Biolab, Merck) and Bacteriological agar (Biolab, Merck). *Oenococcus oeni* strains were cultivated on MRST agar containing MRS and 20 g/L Bacteriological agar supplemented with 10% preservative free tomato juice (All Gold, South Africa) with the pH adjusted to 5.0 with hydrochloric acid (HCl). All plates contained 50 mg/L Delvocid Instant (DSM Food Specialties, The Netherlands) to prevent the growth of yeasts and 25 mg/L Kanamycin Sulphate (Roche Diagnostics GmbH, Mannheim, Germany) to suppress the growth of acetic acid bacteria. Grape must or

TABLE 1
A list of the strains that were selected from the IWBT culture collection and isolated from spontaneous MLF in Pinotage.

Isolate	Species	Isolate	Species
B69	*O. oeni*	2.1	*L. plantarum*
D59	*O. oeni*	14	*L. plantarum*
D60	*O. oeni*	14.1	*L. plantarum*
E53	*O. oeni*	56	*L. plantarum*
J65	*O. oeni*	65	*L. plantarum*
M69	*O. oeni*	66.1	*L. plantarum*
N73	*O. oeni*	68	*L. plantarum*
W56	*O. oeni*	69	*L. plantarum*
W75	*O. oeni*	70	*L. plantarum*
W77	*O. oeni*	71	*L. plantarum*
A1	*O. oeni*	71.1	*L. plantarum*
A2	*O. oeni*	73.1	*L. plantarum*
B1	*O. oeni*	75	*L. plantarum*
C1	*O. oeni*	76.2	*L. plantarum*
S1*	*O. oeni*	77.1	*L. plantarum*
S2*	*O. oeni*	78.1	*L. plantarum*
S3*	*O. oeni*	80.2	*L. plantarum*
S4*	*O. oeni*	107	*L. plantarum*
S5*	*O. oeni*	109	*L. plantarum*
S6*	*O. oeni*		
S7*	*O. oeni*		
S8*	*O. oeni*		
S9*	*O. oeni*		

* Strains isolated from spontaneous MLF in Pinotage.

wine samples were serially diluted ten-fold in sterile water and plated. All LAB were anaerobically cultivated by using Microbiology Anaerocult sheets in anaerobic jars (Merck, Darmstadt, Germany) and incubated at 30ºC for four to seven days depending on the growth of the microorganisms. Colonies were also inspected using light microscopy to investigate the cell morphology.

The culturing of the LAB strains prior to inoculation in the wine were as follow: *L. plantarum* and *O. oeni* strains were grown at 30ºC on MRS agar and MRST agar plates, respectively, until single colonies were clearly distinguishable. After growth on the agar plates, *L. plantarum* strains were grown at 30ºC in MRS broth for two days and *O. oeni* strains were grown in filter-sterilised MRS broth supplemented with 20% preservative free apple juice (Ceres, South Africa) (MRSA) and the pH adjusted to 5.2 with HCl for four days. To ensure survival in the wine after inoculation, strains grown in MRS and MRSA broth were inoculated in an adaptation medium at 1.5% (v/v) and incubated at 30ºC for two days. The adaptation medium consisted of (concentrations indicated in brackets): MRS (Biolab, Merck; 50 g/L), D(-)-fructose (Merck; 40 g/L), D(+)-glucose (Merck; 20 g/L), L(-)-malic acid (Sigma; 4 g/L), Tween 80 (Merck; 1 g/L), absolute ethanol (4% v/v) and with the pH adjusted to 4.6 using potassium hydroxide (KOH) pellets (Saarchem, Merck). The adaptation medium for *O. oeni* strains were filter-sterilised using a 0.22 µm syringe filter and also supplemented with 20% preservative free apple juice. After two days in the adaptation medium, the cultures were centrifuged at 8000 rpm for 10 min and the pellet rehydrated in water before inoculation in the wine at approximately 10^6 CFU/mL.

Screening in synthetic wine medium

For evaluation in the synthetic wine medium, *L. plantarum* strains were cultivated at 30ºC in MRS broth for two days. *Oenococcus oeni* strains were cultivated at 30ºC for four days in filter-sterilised MRSA broth. The ability of the bacterial strains to degrade malic acid was evaluated in a synthetic wine medium adapted from Ugliano *et al*. (2003). The media was prepared with the following adaptations: the pH of the medium was adjusted to 3.4 with KOH pellets and the ethanol concentration was adjusted to 14% (v/v) using absolute ethanol (Saarchem, Merck). No glycoside extract was added and 0.056 g/L $MnSO_4.H_2O$ was added as a substitute for $MnSO_4$. The media was filtered through a 0.45 µm syringe filter (Lasec), followed by filtration through a 0.22 µm syringe filter. The pre-cultured bacteria included 19 *L. plantarum* and 23 *O. oeni* strains and were inoculated at 1.5% (v/v) (approximately 10^6 CFU/mL) in the synthetic wine medium and incubated at 20-22ºC under static anaerobic conditions to undergo MLF. Cell counts were monitored at three different stages during MLF by conducting plate counts on MRS and MRST agar plates that were anaerobically incubated at 30ºC. The malic acid concentration was determined with a malic acid enzymatic assay (Roche, Boehringer Mannheim, Germany) on day seven of MLF.

Molecular detection of genes involved in biogenic amine production

DNA preparation

For DNA preparation purposes, the *L. plantarum* and *O. oeni* strains were grown at 30ºC in MRS or MRSA broth, respectively, supplemented with 1.5% (w/v) glycine (Saarchem, Merck) for 24 hours prior to isolation. The genomic DNA from *L. plantarum* and *O. oeni* strains were isolated as described by Lewington *et al*. (1987). The DNA samples were stored at -20ºC. The quantification of DNA was performed spectrophotometrically using a NanoDrop® ND-1000 (NanoDrop Technologies, Inc., Wilmington, USA).

Genetic screening for enzyme-encoding genes using PCR

Lactobacillus plantarum and *O. oeni* strains that showed potential in the synthetic wine medium were screened for the genes that encode for the histidine- (HDC), tyrosine- (TDC) and ornithine decarboxylase (ODC) enzymes. This was done with a multiplex-PCR method described by Marcobal *et al*. (2005). The primer sets for the decarboxylase genes can be seen in Table 2. The 50 µL reaction mixture contained 100 ng template DNA, 0.3 µM of primer set JV16HC/JV17HC (Whitehead Scientific, South Africa), 1 µM of primer set 3/16 and 2 µM of primer set P1-rev/P2-for, 200 µM dNTP's (Takara, Separations), 1.75 µM $MgCl_2$ (Supertherm, Southern Cross Biotechnology), 1x PCR buffer (Supertherm, Southern Cross Biotechnology) and 1.5 U DNA Polymerase (Supertherm, Southern Cross Biotechnology).

Lactobacillus 30a (histamine- and putrescine-producing LAB strain) and *Lactobacillus brevis* M58 (tyramine producing LAB strain) were selected as positive controls in this PCR reaction (Downing, 2003; Marcobal *et al*., 2005). A negative control containing no bacterial DNA template was included in the PCR reaction. The PCR reaction conditions were as described in Table 3. All PCR reactions were done using a T3 Thermocycler (Whatman Biometra GmbH, Germany). The PCR products were analysed by gel electrophoresis in 1.5% (w/v) agarose (Whitehead Scientific) gels containing 0.2 µg/mL ethidium bromide (Sigma-Aldrich). Gels were run at 85V in a 1x TAE buffer (100 mM Tris-Cl, 1 mM EDTA, pH 8, 20 mM acetate) for approximately 45 min. Ultraviolet transillumination was used for visualising DNA fragments and an Alpha Imager (Alpha Innotech Corporation, San Leandro, California) used for documenting the image. Lambda DNA (Roche) digested with *BstE* II (Roche) was used as the standard molecular weight marker.

Potential starter strains that were able to degrade malic acid in the synthetic wine medium and did not possess any of the genes associated with biogenic amine formation were genetically screened for the presence of enzymes associated with alteration of the sensory profile during MLF, including the production of volatile sulphur compounds, as well as the potential production of ethyl carbamate. The enzymes of interest that were screened for with PCR and gene-specific primers are listed in Table 2. The PCR reactions were carried out using isolated genomic DNA or a single bacterial colony as DNA template. A negative control contained no bacterial template. The gene-specific primer sets for *O. oeni* and/or *L. plantarum* and their applications are listed in Table 2.

TABLE 2
Nucleotide sequences of primer sets for the detection of the genes encoding enzymes of importance to winemaking.

Primer name	Primer sequence (5'-3')	Organism	Positive control(s)	Application	Reference
JV16HC	AGATGGTATTGTTTCTTATG	*L. plantarum* *O. oeni*	*Lactobacillus* 30a	histidine decarboxylase	Marcobal et al. (2005)
JV17HC	AGACCATACACCATAACCTT				
3	GTNTTYAAYGCNGAYAARACNTAYTTYGT		*Lactobacillus* 30a	ornithine decarboxylase	Marcobal et al. (2005)
16	TACRCARAATACTCCNGGNGGRTANGG				
P1-rev	CCRTARTCNGGNATAGCRAARTCNGTRTG		*Lactobacillus brevis* M58	tyrosine decarboxylase	Marcobal et al. (2005)
P2-fwd	GAYATNATNGGNATNGGNYTNGAYCARG				
BGL-1	GTGACTATGGTAGAGTTTCC	*L. plantarum O. oeni*	*L. plantarum* 14.1	*β*-glucosidase	Spano et al. (2005)
BGL-2	TCAAAACCCATTCCGTTCCCCA		*L. plantarum* 76.2		
PAD-1	AARAAYGAYCAYACYRTTGATTACC	*L. plantarum O. oeni*	*L. plantarum* 14.1	phenolic acid decarboxylase	Mtshali et al. (2010)
PAD-3	TTCTTCWACCCAYTTHGGGAAGAA		*L. plantarum* 66.1		
Clase-1	TTACGBCGSACRATGATGTTTGT	*L. plantarum O. oeni*	*L. plantarum* 14.1	citrate lyase	Mtshali et al. (2010)
Clase-2	TATTTTTCAATGTAATTDCCCTCC		*L. plantarum* 66.1		
Est-1	GCTAATTTGTAACCGTATCCGCC	*L. plantarum*	*L. plantarum* 14.1	putative esterase	Mtshali et al. (2010)
Est-2	CGCGCATGTTAACTTTTAGTAGAAC		*L. plantarum* 76.2		
Est-O-1	ATGGCATTTTTAGAAGTTAATTATTATTCACG	*O. oeni*	*O. oeni* 1098	predicted esterase	Lerm (2010)
Est-O-2	CTATGACAAACGTTTTTCTGCTTGATAATT				
Prt-1	GCATGGCTAATAAATCATTAATCAAAG	*L. plantarum*	*L. plantarum* 14.1	serine protease HtrA	Mtshali et al. (2010)
Prt-2	GCTTAGTTACTTTGTTTAGTTAACGTTTTG		*L. plantarum* 76.2		
Prt-O-1	GTGACTGAAGAACAAGACCAAGGAAAAAC	*O. oeni*	*O. oeni* 1098	trypsin-like serine protease	Lerm (2010)
Prt-O-2	TTATTGTTTCAAAGTTTCAGTCATCTTAACCTT				
pepC-for	GGNCGTTGYTGGATGTTYGCBGCHTTRAAYAC	*L. plantarum* *O. oeni*	*Lactobacillus brevis* 117.2	cysteine aminopeptidase	Mtshali (2010)
pepC-rev	TTADGCHAAWGHWCCCATTGGRTCCCAHGG				
pepI-for	CCYGGTGGHAABCAYGARTAYTGGGAARAC	*L. plantarum* *O. oeni*	*Lactobacillus brevis* 117.2	proline iminopeptidase	Mtshali (2010)
pepI-rev	GCRTTRTCDATCATGTGRTGRTGRCCRCCDT				
pepM-for	GGHTTTGAAGGHTAYAARTATKCNACBTGTGT	*L. plantarum* *O. oeni*	*Lactobacillus brevis* 117.2	methionine aminopeptidase	Mtshali (2010)
pepM-rev	AYCATBGGTTCRATNGTAATBGTCATVCC				
pepN-for	ATGGAAAACTGGGGNYTDGTNACHTAYCG	*L. plantarum* *O. oeni*	*Lactobacillus brevis* 117.2	membrane alanine aminopeptidase	Mtshali (2010)
pepN-rev	ACNRCNGGRTADCCNGGYTGTTCVARCCANG				
citD-f	ATGGAAATTAARAMAACKGCAKTMGC	*L. plantarum* *O. oeni*	*Lactobacillus brevis* 117.2 *O. oeni* B45	citrate lyase γ-subunit	Mtshali et al. (2010)
citD-r	GCYGCYGTAATRGTYGKYGCYTTWAT				
citF-a	ATGGYATGACRATTTCWTTYCAYCAYCA	*L. plantarum* *O. oeni*	*Lactobacillus brevis* 117.2 *O. oeni* B45	citrate lyase α-subunit	Mtshali et al. (2010)
citF-b	ATCAATVAHBSWRCCRTCRCGRTAYTC				

TABLE 2 (CONTINUED)

Primer name	Primer sequence (5'-3')	Organism	Positive control(s)	Application	Reference
maeP-1	ATGGGTGTTTTTTGGACATCG	*O. oeni*	*O. oeni* B45	putative citrate transporter	Mtshali (2010)
maeP-2	TCAAATAAAGTTGATGATACTCATTA				
alsD-Oe1	ATGAAAGATTTAACAAAAGCTTATC	*O. oeni*	*O. oeni* B45	α-acetolactate reductase	Mtshali (2010)
alsD-Oe2	TTATTCTGTCTTTTCAATCGCTT				
alsS-Oe1	ATGACAGAAAAGAAACGTTTTGGG	*O. oeni*	*O. oeni* B45	α-acetolactate synthase	Mtshali (2010)
alsS-Oe2	TTAATCCATATCTCCTTCGATCAATTC				
alsS-deg1	GGTTAYGAYSCSRTYGAATATGARCCNCG	*L. plantarum* *O. oeni*	*Lactobacillus brevis* 117.2 *O. oeni* B45	α-acetolactate synthase	Mtshali (2010)
alsS-deg2	ATTTCYTCTTGRAAYTTRACCATRTCGTA				
ADI-for	CAYGCNATGATGCAYYTNGAYACNGT	*L. plantarum* *O. oeni*	*Lactobacillus florum* 29.1	arginine deiminase	Araque et al. (2009)
ADI-rev	GTRTTNSWNCCRTCRTTCCAYTGYTC				
OTC-for	ATGCAYTGYYTNCCNGCNTTYCAYGA	*L. plantarum* *O. oeni*	*Lactobacillus florum* 29.1	ornithine transcarbamylase	Araque et al. (2009)
OTC-rev	CCNARNGTNGCNGCCATDATNGCYTT				
CK-for	CAYGGNAAYGGNCCNCARGTNGGNAA	*L. plantarum* *O. oeni*	*O. oeni* B45	carbamate kinase	Araque et al. (2009)
CK-rev	CKNCKNYANCCNCKNCCNGCRTCYTC				
Sams-1	GAAMGMCAYTTATTTACDTCGA	*L. plantarum*	*Lactobacillus brevis* 117.2	S-adenosylmethionine synthase	Mtshali (2010)
Sams-2	AATBCCAGCWGGBCGYAARTCAAA				
SadsOE-F	ATGAAAAAGTTTTTTACGAGTGAGTCGG	*O. oeni*	*O. oeni* B45	S-adenosylmethionine synthase	Knoll et al. (2010)
SadsOE-R	TTAATTTGCCAAAAGAGCTTTAATTTTTTC				
CBL-OE-F	ATGACAGAATCCGATTGG	*O. oeni*	*O. oeni* B45	cystathionine *β*-lyase	Knoll et al. (2010)
CBL-OE-R	TTAATCCTCCAATGC				
CBGL-deg1	ATGAAATTYRAWACMMAAYTWATTCAYGGYGG	*L. plantarum* *O. oeni*	*Lactobacillus brevis* 117.2 *O. oeni* B45	cystathionine *β*-lyase/ γ-lyase	Mtshali (2010)
CBGL-deg2	ACCVACHGAKARRCGRATYAGYTCGTCTT				
CGL-OE-F	ATGAAATTCAATACAAAACTTATTCATG	*O. oeni*	*O. oeni* B45	cystathionine γ-lyase	Knoll et al. (2010)
CGL-OE-R	CTAAATCTTGCTGAATGAC				
GSH-OE-F	ATGAAAAACCAGCAATATG	*O. oeni*	*O. oeni* B45	glutathione reductase	Knoll et al. (2010)
GSH-OE-R	TTACAATTGGCCGGCTG				

N: G, A, T or C; R: G or A; M: A or C; Y: T or C; K: G or T; W: A or T; D: G, A or T; S: G or C; H: A, C or T; B: G, T or C; V: G, C or A.

TABLE 3
Thermal cycling conditions of the PCR reactions used for the detection of the genes associated with biogenic amine formation and genetic screening of the enzymatic profiles of wine LAB.

Primer pair	Gene size (bp)	T_{Di} (ºC), time	Main cycling conditions				T_{Ef} (ºC), time
			Number of cycles	T_D (ºC), time	T_A (ºC), time	T_E (ºC), time	
JV16HC/JV17HC	367						
3/16	1446	94ºC, 10 min	30	95ºC, 30 sec	52ºC, 30 sec	72ºC, 2 min	72ºC, 10 min
P1-rev/P2-fwd	924						
BGL-1/BGL-2	1392	94ºC, 5 min	30	94ºC, 1 min	50ºC, 40 sec	72ºC, 62 sec	72ºC, 10 min
PAD-1/PAD-3	219	94ºC, 2 min	35	94ºC, 40 sec	50ºC, 1 min	72ºC, 30 sec	72ºC, 5 min
Clase-1/Clase-2	897	94ºC, 3 min	35	94ºC, 30 sec	54ºC, 1 min	72ºC, 1 min	72ºC, 10 min
Est-1/Est-2	1020	94ºC, 5 min	30	94ºC, 1 min	53ºC, 30 sec	72ºC, 1 min	72ºC, 10 min
Est-O-1/Est-O-2	804	94ºC, 5 min	35	94ºC, 1 min	50ºC, 1 min	72ºC, 1 min	72ºC, 10 min
Prt-1/Prt-2	1263	94ºC, 5 min	30	94ºC, 1 min	55ºC, 30 sec	72ºC, 1 min	72ºC, 10 min
Prt-O-1/Prt-O-2	1278	94ºC, 5 min	35	94ºC, 1 min	52ºC, 90 sec	72ºC, 90 sec	72ºC, 10 min
pepC-for/pepC-rev	1142	94ºC, 5 min	30	94ºC, 1 min	54ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
pepI-for/pepI-rev	737	94ºC, 5 min	30	94ºC, 1 min	53ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
pepM-for/pepM-rev	443	94ºC, 5 min	30	94ºC, 1 min	49ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
pepN-for/pepN-rev	569	94ºC, 5 min	30	94ºC, 1 min	49ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
citD-f/citD-r	245	94ºC, 3 min	35	94ºC, 45 sec	54ºC, 30 sec	72ºC, 1 min	72ºC, 5 min
citF-a/citF-b	1331	94ºC, 5 min	35	94ºC, 1 min	49ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
maeP-1/maeP-2	984	94ºC, 5 min	35	94ºC, 1 min	49ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
alsD-Oe1/alsD-Oe2	717	94ºC, 5 min	35	94ºC, 1 min	49ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
alsS-Oe1/alsS-Oe2	1683	94ºC, 5 min	35	94ºC, 1 min	49ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
alsS-deg1/alsS-deg2	620	94ºC, 5 min	30	94ºC, 1 min	55ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
ADI-for/ADI-rev	266	94ºC, 5 min	30	94ºC, 1 min	55ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
OTC-for/OTC-rev	181	94ºC, 5 min	30	94ºC, 1 min	49ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
CK-for/CK-rev	343	94ºC, 5 min	30	94ºC, 1 min	49ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
Sams-1/Sams-2	1080	94ºC, 3 min	35	94ºC, 30 sec	54ºC, 1 min	72ºC, 1 min	72ºC, 10 min
SadsOE-F/SadsOE-R	1167	94ºC, 2 min	30	94ºC, 1 min	48ºC, 1 min	72ºC, 1 min	72ºC, 5 min
CBL-OE-F/CBL-OE-R	1134	94ºC, 2 min	30	94ºC, 1 min	45ºC, 1 min	72ºC, 1 min	72ºC, 5 min
CBGL-deg1/CBGL-deg2	1080	94ºC, 5 min	30	94ºC, 1 min	49ºC, 45 sec	72ºC, 1 min	72ºC, 10 min
CGL-OE-F/CGL-OE-R	1137	94ºC, 2 min	30	94ºC, 1 min	46ºC, 1 min	72ºC, 1 min	72ºC, 5 min
GSH-OE-F/GSH-OE-R	1338	94ºC, 2 min	30	94ºC, 1 min	50ºC, 1 min	72ºC, 1 min	72ºC, 5 min

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

The PCR reaction mixtures are listed in Table 4. All PCR reactions were done as previously described. The PCR products were analysed by gel electrophoresis in 1 to 3% (w/v) agarose (Whitehead Scientific) gels containing 0.5x GelRed (Biotium). Gels were run at 80V in a 1x TAE buffer (100 mM Tris-Cl, 1 mM EDTA, pH 8, 20 mM acetate) for approximately 45-60 minutes. Visualisation of the DNA fragments was as previously described. A 100 bp DNA Molecular Weight Marker XIV (Roche) was used as the standard molecular weight marker.

Small-scale vinification procedures

Alcoholic fermentation procedures

Vinifications were conducted in Pinotage sourced from the Wellington region, South Africa. Half a ton of grapes were crushed and destemmed. Before the onset of AF, representative homogenous samples of the grape must were taken to determine the standard must parameters. Sulphur dioxide was added to the must at a concentration of 30 mg/L before the onset of AF to inhibit the growth of indigenous microflora. Lysozyme (DSM Food Specialties, Oenology, France) was added at 0.25 g/L to inhibit indigenous LAB. The grape must was inoculated for batch AF with a commercial strain of *Saccharomyces cerevisiae*, WE372 (Anchor Yeast). Rehydration of the yeast strain was performed according to the manufacturer's specifications. Alcoholic fermentation of the grape must took place in contact with the grape skins. The decrease in sugar levels was measured on a daily basis using a Brix hydrometer to monitor the progression of AF. Alcoholic fermentation took place at 25ºC and the skins were mixed with the juice on a daily basis using a manual punch-down method. Forty-eight hours after yeast inoculation, Nutrivin (Anchor Yeast), a nutrient supplement, was added at 70 g/hL. This provided a nutrient source for the yeast to avoid stuck or sluggish AF. After the completion of AF (less than 1 g/L residual sugar) the wines were pressed with a hydraulic basket press and divided into 2 L glass bottles (sealed with airlocks) to complete spontaneous or induced MLF at 20ºC.

Malolactic fermentation procedures

The individual *O. oeni* and *L. plantarum* cultures were evaluated in duplicate in Pinotage (Table 5). The cultures were inoculated after the completion of AF, referred to as sequential inoculation. An *O. oeni* commercial malolactic starter culture, Lalvin VP41® (Lallemand) was used as control fermentation. A treatment receiving no inoculation was used as a spontaneous fermentation control. The commercial MLF starter culture used during the small-scale vinifications was inoculated according to the instructions of the manufacturer at the recommended maximum dosage. All MLF treatments received a nutrient supplement, Optimalo'Plus (Lallemand), at a dosage of 20 g/hL 48 h after inoculation of the bacterial culture.

After the completion of MLF, the wines were removed from the yeast lees and SO_2 was added to all wines prior to bottling to achieve a total SO_2 concentration of 80 mg/L.

Standard wine analysis

Determination of the standard parameters of the juice and wine were performed with a WineScan FT120 spectrophotometer (FOSS Analytical, Denmark) (according to the method described by Louw *et al*., 2009). Duplicate scans were obtained for each sample. The standard parameters determined for the juice include: pH, volatile- and total acid concentration, reducing sugars, malic acid concentration, glucose and fructose concentration and density. Wine parameters include: pH, volatile- and total acidity, malic-

TABLE 4
PCR mixtures for the genetic detection of genes that encode for aroma- and wholesomeness-related enzymes in LAB.

Enzyme	Organism	Size (µL)	Concentration					
			Template DNA	Each Primer (µM)[1]	dNTP's (µM)[2]	$MgCl_2$ (mM)[3]	Buffer[3]	DNA Polymerase (U)[3]
β-glucosidase	*L. plantarum* *O. oeni*	50	100 ng	0.5	200	1.5	1x	1.25
PAD	*L. plantarum* *O. oeni*	50	100 ng	0.4	200	-	1x	1[4]
Citrate lyase	*L. plantarum* *O. oeni*	25	100 ng	0.4	250	0.75	1x	2
Esterase, Protease	*L. plantarum*	50	100 ng	0.4	250	1.5	1x	1.25
Esterase, Protease	*O. oeni*	50	100 ng	2	250	1.5	1x	1.25
Remaining enzymes	*L. plantarum* *O. oeni*	25	Bacterial colony	6	250	1.5	1x	1.25

[1] Whitehead Scientific, South Africa
[2] Takara, Seperations
[3] Supertherm, Southern Cross Biotechnology
[4] ExTaq, Takara Biomedical

TABLE 5
The malic acid concentration measured after seven days in the synthetic wine medium inoculated with the different LAB strains. The synthetic wine medium contained an initial malic acid concentration of 3.5 g/L.

LAB	Malic acid (g/L)	LAB	Malic acid (g/L)
O. oeni B69	1.08	*L. plantarum* 2.1	0.71
O. oeni D59	0.73	*L. plantarum* 14	0.83
O. oeni D60	0.28	*L. plantarum* 14.1 *	0.00
O. oeni E53 *	0.28	*L. plantarum* 56 *	0.00
O. oeni J65 *	0.81	*L. plantarum* 65	1.32
O. oeni M69	1.08	*L. plantarum* 66.1 *	0.27
O. oeni N73	1.10	*L. plantarum* 68 *	0.02
O. oeni W56	1.09	*L. plantarum* 69	0.17
O. oeni W75	1.11	*L. plantarum* 70	0.08
O. oeni W77	0.98	*L. plantarum* 71	0.72
O. oeni A1	0.98	*L. plantarum* 71.1 *	0.17
O. oeni A2 *	0.58	*L. plantarum* 73.1	0.41
O. oeni B1 *	0.49	*L. plantarum* 75	1.08
O. oeni C1	1.26	*L. plantarum* 76.2	0.53
O. oeni S1	0.49	*L. plantarum* 77.1	1.10
O. oeni S2	0.35	*L. plantarum* 78.1 *	0.24
O. oeni S3	0.95	*L. plantarum* 80.2	1.04
O. oeni S4	0.28	*L. plantarum* 107 *	0.05
O. oeni S5 *	0.25	*L. plantarum* 109	0.65
O. oeni S6 *	0.32		
O. oeni S7	0.55		
O. oeni S8	0.40		
O. oeni S9	1.05		

* strains selected for evaluation in Pinotage

and lactic acid, glucose and fructose, ethanol and glycerol. Sulphur dioxide (total and free) analysis was carried out using the Metrohm titration unit (Metrohm Ltd., Switzerland). Malolactic fermentation in the wine was monitored by obtaining the malic- and lactic acid concentrations on a regular basis (at the start of MLF and every seven days thereafter) until its completion (taken as the point when the malic acid concentration is equal to or lower than 0.3 g/L). This was done by Fourier-transform Infrared Spectroscopy (FT-IR) (WineScan FT120, FOSS Analytical, Denmark) as well as determination of the malic acid concentration with an enzymatic assay (Roche, Boehringer Mannheim, Germany).

RESULTS

Screening in synthetic wine medium

Nineteen *L. plantarum* and 23 *O. oeni* strains were identified and selected for evaluation in the synthetic wine medium. The screening process in the synthetic wine medium was used as an indication of the possible survival and performance of the potential starter strains in the actual wine environment. Table 5 lists the LAB strains and the corresponding malic acid concentrations measured in the synthetic wine medium after seven days of MLF at 20-22ºC. The *L. plantarum* strains performed the best and nine of the strains were able to convert the initial malic acid concentration of 3.5 g/L to below 0.3 g/L in seven days. Seven *O. oeni* and seven *L. plantarum* strains that showed potential in the synthetic medium were selected for further characterisation. Selection was not only based on the performance of the potential starter strains in the synthetic wine medium with regards to malic acid degradation, but also the ability of the strains to proliferate and maintain sufficient population numbers during the completion of MLF (these strains are highlighted in Table 6).

Genes involved in biogenic amine production

The seven *L. plantarum* and seven *O. oeni* strains that successfully degraded malic acid or displayed potential in the synthetic medium, were screened for the genes that encode for the amino acid decarboxylase enzymes responsible for biogenic amine formation. The primers amplified single products of 367 bp and 1446 bp for *Lactobacillus* 30a, corresponding to fragments of the *hdc* and *odc* genes, respectively and a product of 924 bp for *Lactobacillus brevis* M58, corresponding to a fragment of the *tdc* gene. Of the 14 selected strains that were screened (results not shown), none of the strains contained *hdc*, *odc* or *tdc* gene fragments.

PCR detection of genes encoding enzymes of enological interest

Genetic screenings of the enzyme-encoding genes were done for the 14 selected LAB strains that were negative for the genes

TABLE 6
Cell counts (CFU/mL) of the individual *L. plantarum* and O. oeni strains as recorded at the start and different stages of MLF in the synthetic wine medium.

		Days after inoculation					Days after inoculation		
		0	2	6			0	2	6
L. plantarum	2.1	$5.4x10^7$	$2.4x10^7$	$1.5x10^6$	***O. oeni***	B69	$2.9x10^7$	$1.0x10^6$	$2.0x10^4$
	14	$4.2x10^7$	$3.0x10^6$	$8.0x10^5$		D59	$1.0x10^5$	$1.0x10^5$	$1.0x10^4$
	14.1*	$3.5x10^7$	$1.3x10^7$	$6.2x10^6$		D60	$4.3x10^8$	$7.0x10^7$	$1.0x10^7$
	56*	$4.7x10^7$	$1.5x10^7$	$2.0x10^6$		E53*	$1.9x10^8$	$3.4x10^7$	$7.0x10^5$
	65	$8.3x10^6$	$4.0x10^5$	$2.4x10^5$		J65*	$2.6x10^8$	$5.3x10^7$	$5.0x10^6$
	66.1*	$5.5x10^7$	$2.5x10^7$	$9.9x10^5$		M69	$3.0x10^8$	$5.0x10^7$	$5.0x10^6$
	68*	$3.6x10^7$	$1.7x10^7$	$2.9x10^6$		N73	$1.2x10^7$	$1.0x10^6$	$3.0x10^4$
	69	$4.6x10^7$	$2.7x10^7$	$1.3x10^6$		W56	$4.7x10^7$	$7.0x10^7$	$1.0x10^6$
	70	$3.6x10^7$	$1.0x10^7$	$3.0x10^6$		W75	$1.1x10^5$	$1.0x10^5$	$1.0x10^4$
	71	$2.2x10^7$	$3.2x10^6$	9.0x105		W77	$1.0x10^5$	$1.0x10^5$	$1.0x10^4$
	71.1*	$4.4x10^7$	$4.7x10^6$	$2.3x10^6$		A1	$8.1x10^7$	$8.0x10^7$	$6.9x10^6$
	73.1	$4.0x10^7$	$9.9x10^6$	$7.5x10^6$		A2*	$9.0x10^7$	$7.9x10^7$	$1.0x10^6$
	75	$3.0x10^6$	$1.2x10^6$	$5.9x10^5$		B1*	$2.8x10^8$	$1.5x10^8$	$1.0x10^5$
	76.2	$6.2x10^7$	$2.2x10^7$	$5.4x10^6$		C1	$1.6x10^8$	$1.2x10^8$	$2.0x10^5$
	77.1	$2.4x10^7$	$2.3x10^6$	$1.4x10^6$		S1	$1.5x10^8$	$8.6x10^7$	$7.5x10^5$
	78.1*	$3.6x10^7$	$9.4x10^6$	$1.9x10^6$		S2	$1.5x10^8$	$1.3x10^8$	$5.0x10^5$
	80.2	$3.6x10^7$	$3.6x10^6$	$2.9x10^6$		S3	$2.0x10^8$	$1.5x10^8$	$3.0x10^5$
	107*	$5.8x10^7$	$2.2x10^6$	$4.2x10^4$		S4	$2.6x10^8$	$1.3x10^8$	$1.0x10^5$
	109	$3.3x10^7$	$1.4x10^7$	$2.6x10^6$		S5*	$4.8x10^7$	$3.3x10^8$	$8.0x10^5$
						S6*	$5.0x10^7$	$2.0x10^7$	$9.0x10^5$
						S7	$1.7x10^8$	$1.2x10^8$	$3.0x10^5$
						S8	$1.0x10^8$	$9.6x10^7$	$4.0x10^5$
						S9	$1.7x10^8$	$1.2x10^8$	$2.0x10^5$

* strains selected for evaluation in Pinotage

associated with biogenic amine formation. The enzymes of interest that were screened for and could potentially influence the aroma profile, included β-glucosidase, PAD, citrate lyase, esterase, protease, peptidases and enzymes related to the production of volatile sulphur compounds. Other enzymes of interest included those that play a role in the production of ethyl carbamate. The results of the genetic screening of the enzymes for *L. plantarum* and *O. oeni* are shown in Tables 7 and 8, respectively.

Malolactic fermentation

Must and wine composition before inoculation

The chemical composition of the Pinotage grape must utilised for evaluation of the cultures were as follow (concentrations indicated in brackets): total sugar (309 g/L), malic acid (1.79 g/L), total acid (5.72 g/L) and pH 3.97. The standard wine parameters after AF and before inoculation for MLF were as follow: pH 3.89, volatile acidity (0.45 g/L), total acidity (5.28 g/L), malic acid (1.76 g/L), lactic acid (0 g/L) and ethanol (15.82% v/v). The wine was fermented until the residual sugar concentration was determined as being less than 1 g/L. The Pinotage grapes were harvested at a very high sugar level which resulted in a high alcohol concentration.

Evaluation of fermentation capability

The isolates that were able to degrade malic acid in the synthetic wine medium, were able to maintain sufficient population numbers and were able to proliferate in the adaptation medium prior to inoculation in the wine, were selected for evaluation in Pinotage (Table 5). The commercial culture Lalvin VP41® (Lallemand) was included to compare the fermentation performance of the selected LAB strains with that of a commercial product. By monitoring a spontaneous fermentation control receiving no inoculation, this could provide an indication of the contribution of the indigenous LAB population to the fermentation process.

Of the seven *L. plantarum* strains that were evaluated, only three completed MLF (Fig. 1), while the remaining four strains did not complete MLF. The three strains were *L. plantarum* 14.1, 56 and 107. The control *O. oeni* culture VP41 showed the best performance and completed the fermentation in 23 days. Of the best performing *L. plantarum* strains, *L. plantarum* 107 finished MLF in 30 days, compared to strains 56 and 14.1 which required 44 days. Despite the fact that *L. plantarum* 107 cell counts showed the most drastic decrease over the course of the fermentation (Table 9), a faster fermentation rate was evident. The spontaneous fermentation never completed MLF. Microbiological analysis (Table 9) showed that all

the treatments were inoculated at approximately $2x10^6$ to $4x10^6$ CFU/mL. *Lactobacillus plantarum* cell counts stayed constant or showed a slight decrease over the initial fermentation period, followed by a steady decline towards the end of fermentation. Cell counts of VP41 stayed constant over the course of the fermentation. Cell numbers eventually decreased to approximately 10^4 CFU/mL after the completion of MLF.

Oenococcus oeni W56 displayed diminished growth on the agar medium, in broth and the adaptation medium, compared to the other strains. For this reason, *O. oeni* W56 was not included in the evaluations of the strains in Pinotage. Four of the six *O. oeni* strains were able to complete MLF (Fig. 2). These four *O. oeni* isolates, S5, S6, J65 and E53, completed MLF in nine days, showing a faster fermentation rate than VP41, which took 16 days to complete fermentation. Microbiological analysis showed that the *O. oeni* isolates were inoculated at approximately

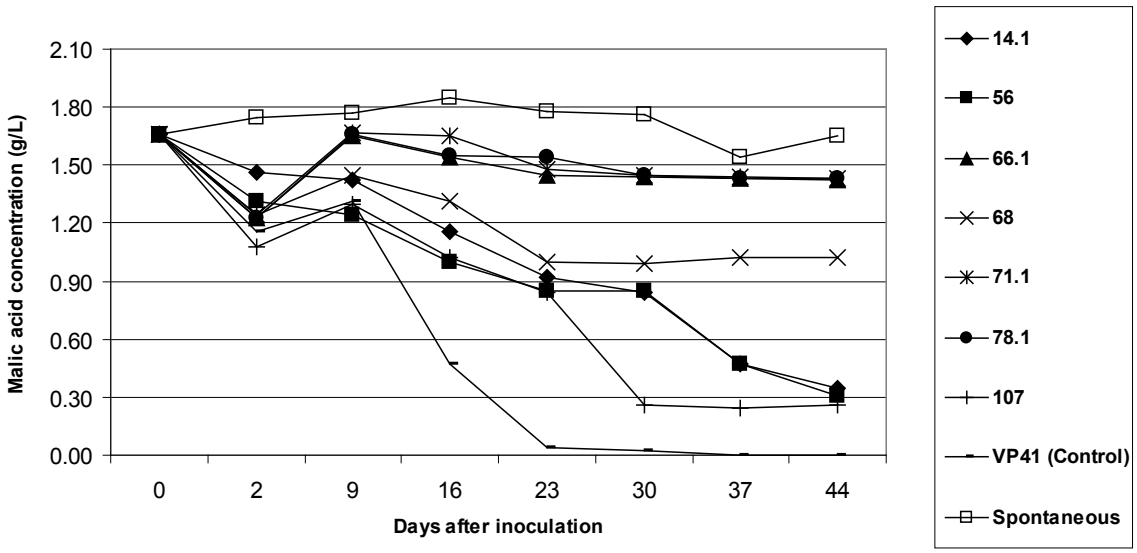

FIGURE 1

A fermentation graph showing the evolution of malic acid (g/L) in the MLF treatments evaluating the individual *L. plantarum* strains in Pinotage. Fermentations were monitored from the start of MLF on a weekly basis. Data shown indicate the average changes in the malic acid concentration (g/L) of each treatment repeated in duplicate. The relative standard deviation (RSD) is less than 10% between fermentation repeats.

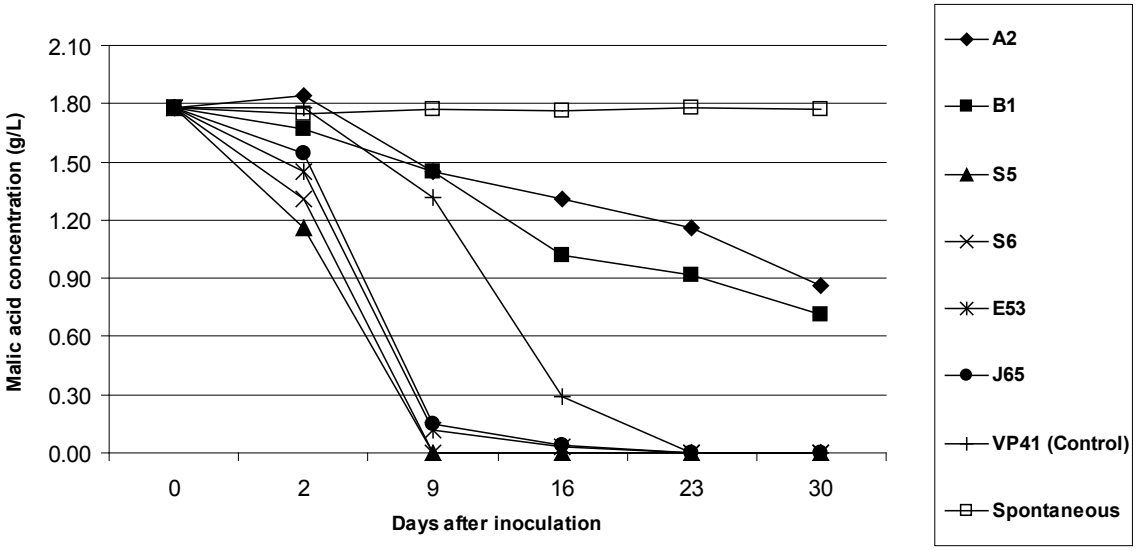

FIGURE 2

A fermentation graph showing the evolution of malic acid (g/L) in the MLF treatments evaluating the individual *O. oeni* strains in Pinotage. Fermentations were monitored from the start of MLF on a weekly basis. Data shown indicate the average changes in the malic acid concentration (g/L) of each treatment repeated in duplicate. The RSD is less than 10% between fermentation repeats.

10^7 CFU/mL (Table 9) and VP41 at $2x10^6$ CFU/mL. This could be a possible contributing factor to the faster fermentation rate displayed by the isolates compared to that of the control culture. Cell counts stayed constant during the early stages of MLF and slightly increased over the course of the fermentation. *Oenococcus oeni* B1 and J65 were the only treatments of which the bacterial population decreased to 10^6 CFU/mL at the end of fermentation.

None of the LAB isolates resulted in volatile acidity concentrations exceeding the sensory threshold value of 0.7 g/L (Jackson, 2008) (results not shown). The seven *L. plantarum* strains showed similar final volatile acidity concentrations (±0.50 g/L). *Oenococcus oeni* strains A2, B1 and J65 displayed slightly lower volatile acidity concentrations (±0.48 g/L) compared to *O. oeni* S5, S6 and E53 (0.57 g/L).

DISCUSSION

This study focused on characterising *O. oeni* and *L. plantarum* strains for possible use in a MLF starter culture. Strains were first selected after characterisation in a synthetic wine matrix, which resulted in 13 potential MLF strains. These strains were then evaluated in wine which eliminated certain strains as they did not perform MLF satisfactorily. There are various other studies that also make use of synthetic or wine-like media for the characterisation of LAB strains. Capozzi *et al*. (2010) made use of two different wine-like media with 11 and 13% (v/v) ethanol, respectively. Despite the fact that their *O. oeni* strains were isolated from wines with 12.5 and 13.9% (v/v) ethanol, the *O. oeni* strains were unable to survive in the 13% (v/v) ethanol medium. These results are similar to our findings. The *O. oeni* strains seem to find higher ethanol concentrations in the synthetic wine medium to be more challenging, which is in direct contrast to their performance in wine. These findings are supported by Solieri *et al*. (2010), a study in which a lower ethanol concentration of 10% (v/v) allowed the *O. oeni* strains to complete MLF. Potential limitations of the synthetic wine medium are the lack of phenolic compounds and specific nutrients needed for growth. The addition of these compounds is essential because wine LAB, particularly *O. oeni*, are generally inhibited by tannins and phenolic

TABLE 7

The results from the genetic screening of the enzymes of the *L. plantarum* strains. Presence of the gene is indicated with (+) and absence of the gene is indicated with (-).

Strains	*β*-glucosidase	PAD	Esterase	Protease
14.1	+	+	+	+
56	+	+	+	+
66.1	+	+	+	+
68	+	+	+	+
71.1	+	+	+	-
78.1	-	-	+	+
107	+	+	+	+

Strains	Cysteine aminopeptidase	Proline iminopeptidase	Methionine aminopeptidase	Membrane alanine aminopeptidase
14.1	+	+	+	+
56	+	+	-	+
66.1	+	+	+	+
68	+	+	+	+
71.1	+	+	+	+
78.1	+	+	+	+
107	+	+	+	+

Strains	Citrate lyase	Citrate lyase γ-subunit	Citrate lyase α-subunit	α-Acetolactate synthase
14.1	+	+	-	+
56	+	+	+	+
66.1	+	+	+	+
68	+	+	+	+
71.1	+	+	+	+
78.1	+	+	+	+
107	+	-	-	+

Strains	Arginine deiminase	Ornithine transcarbamylase	Carbamate kinase	Cystathionine *β*/γ-lyase	S-adenosylmethionine synthase
14.1	-	+	+	+	-
56	-	+	+	+	+
66.1	-	+	+	+	+
68	-	+	+	+	-
71.1	-	+	+	+	+
78.1	-	+	+	+	-
107	-	+	+	-	+

TABLE 8
The results from the genetic screening of the enzymes of the *O. oeni* strains. Presence of the gene is indicated with (+) and absence of the gene is indicated with (-).

Strains	*β*-glucosidase	PAD	Esterase	Protease
A2	-	-	+	+
B1	-	-	+	+
S5	-	-	+	+
S6	-	-	+	+
E53	-	-	+	+
J65	-	-	+	+
W56	-	-	+	+

Strains	Cysteine aminopeptidase	Proline iminopeptidase	Methionine aminopeptidase	Membrane alanine aminopeptidase
A2	+	-	-	+
B1	+	-	+	+
S5	+	-	-	+
S6	+	-	-	+
E53	+	-	-	+
J65	+	-	-	+
W56	+	+	-	+

Strains	Citrate lyase	Citrate lyase γ-subunit	Citrate lyase α-subunit	Putative citrate transporter
A2	+	+	-	+
B1	+	+	-	+
S5	+	+	-	+
S6	+	+	+	+
E53	+	+	+	+
J65	+	+	-	+
W56	+	-	-	-

Strains	α-Acetolactate reductase	α-Acetolactate synthase	Arginine deiminase	Ornithine transcarbamylase	Carbamate kinase
A2	+	+	+	+	+
B1	+	+	+	+	-
S5	+	+	+	+	+
S6	+	+	+	+	+
E53	+	+	+	+	+
J65	+	+	+	+	+
W56	-	+	-	+	+

Strains	Cystathionine *β*-lyase	Cystathionine γ-lyase	Cystathionine *β*/γ-lyase	S-adenosylmethionine synthase	Glutathione reductase
A2	+	+	+	-	+
B1	+	+	+	+	+
S5	+	+	+	+	+
S6	-	+	+	+	+
E53	-	+	+	+	+
J65	+	+	+	+	+
W56	-	-	+	-	-

acids, while anthocyanins and gallic acid have a stimulatory effect on growth and malolactic activity (Vivas *et al*., 1997; Campos *et al*., 2003). It has been shown that strains of *O. oeni* and some strains of the *Lactobacillus* genera have essential nutrient requirements, particularly with regards to amino acids, but also vitamins and in the case of *O. oeni*, the so-called "tomato juice factor", a pantothenic acid derivative (Fugelsang & Edwards, 1997; Terrade & Mira de Orduña, 2009). These studies and our results highlight the need for a synthetic wine-like medium that better simulates the wine environment and could provide a more accurate indication of the malic acid degradation ability and survival of LAB strains in wine.

The inability to produce biogenic amines is an important characteristic for any strain being considered for use in a starter culture, as biogenic amines have an impact on wine wholesomeness, have several health implications as well as impacting wine aroma. Our results correlate with other studies, although results on *O. oeni* in literature vary. *Oenococcus oeni* has been identified as the main histamine producer (Moreno-Arribas *et al*., 2000) and 60% of the *O. oeni* strains studied by Guerrini *et al*. (2002) were able to produce histamine, while Landete *et al*. (2005) found that although *O. oeni* was the most frequent histamine producer, this species also produced the lowest concentrations and that this production is strain dependent (Rosi *et al*., 2009). In a

TABLE 9
Cell counts (CFU/mL) of the individual LAB isolates and control fermentations as recorded at the start and different stages of MLF in Pinotage. Each enumeration represents the average of duplicate treatments (standard deviations not shown).

		Days after inoculation					
		0	**2**	**9**	**16**	**23**	**30**
L. plantarum	14.1	$2.9x10^6$	$1.2 x10^6$	$1.3 x10^6$	$1.0 x10^6$	$3.2 x10^5$	$1.0 x10^5$
	56	$3.1 x10^6$	$3.0 x10^6$	$1.3 x10^6$	$5.0 x10^5$	$1.3 x10^5$	$8.4 x10^4$
	66.1	$3.6 x10^6$	$3.9 x10^6$	$7.9 x10^5$	$2.4 x10^5$	$7.1 x10^4$	$1.0 x10^4$
	68	$3.0 x10^6$	$2.7 x10^6$	$4.8 x10^5$	$9.0 x10^4$	$1.4 x10^4$	$1.0 x10^4$
	71.1	$3.5 x10^6$	$1.7 x10^6$	$2.9 x10^5$	$1.8 x10^5$	$7.9 x10^4$	$4.0 x10^4$
	78.1	$4.9 x10^6$	$2.4 x10^6$	$2.9 x10^5$	$1.5 x10^5$	$7.6 x10^4$	$2.4 x10^4$
	107	$3.3 x10^6$	$2.7 x10^6$	$1.0 x10^6$	$3.5 x10^4$	$2.7 x10^3$	$1.2 x10^3$
	VP41	$3.2 x10^6$	$1.3 x10^6$	$1.0 x10^6$	$1.2 x10^6$	$2.8 x10^6$	$4.3 x10^6$
		Days after inoculation					
		0	**2**	**9**	**16**		
O. oeni	A2	$2.3x10^7$	$2.1 x10^7$	$2.3 x10^7$	$1.2 x10^7$		
	B1	$2.5 x10^7$	$1.5 x10^7$	$1.5 x10^7$	$8.9 x10^6$		
	S5	$1.9 x10^7$	$1.4 x10^7$	$2.5 x10^7$	$2.3 x10^7$		
	S6	$1.7 x10^7$	$1.3 x10^7$	$2.3 x10^7$	$2.4 x10^7$		
	E53	$1.4 x10^7$	$1.2 x10^7$	$1.3 x10^7$	$1.5 x10^7$		
	J65	$1.7 x10^7$	$1.2 x10^7$	$4.0 x10^6$	$4.0 x10^6$		
	VP41	$2.1 x10^6$	$1.9 x10^6$	$1.7 x10^6$	$3.0 x10^6$		

study of 113 wine-isolated *O. oeni* strains, 12 strains were positive for the biogenic amine-encoding genes histidine decarboxylase (10 strains) and ornithine decarboxylase (two strains) (Coton *et al*., 2010).

Although *Lactobacillus* species have been identified as the main tyramine producers by Moreno-Arribas *et al*. (2000), *L. plantarum* has only recently been implicated in the production of biogenic amines (Manfroi *et al*., 2009). In a screening of 50 wine-isolated *L. plantarum* strains for biogenic amine-encoding genes by Coton *et al*. (2010), it was found that only one strain was positive for the agmatine decarboxylase gene and one strain for the tyrosine decarboxylase gene. Contrary to this, both *L. brevis* and *Lactobacillus hilgardii* have been identified as producers of tyramine, phenylethyl amine and putrescine, respectively (Arena & Manca de Nadra, 2001; Landete *et al*., 2007). In order to totally eliminate the potential for biogenic amine production, screening possible starter cultures for biogenic amine-encoding genes is a quick and efficient method to ensure this.

In order to investigate the potential influence of the potential starter strains on the final wine aroma profile and quality, they were genetically screened for enzymes pertaining to the aroma and flavour profile and wholesomeness of wines undergoing MLF. Some of the biggest differences in the enzymatic profiles of *O. oeni* and *L. plantarum* were the presence or absence of the genes encoding for the enzymes *β*-glucosidase and PAD. Various potentially volatile grape-derived aroma compounds exist, but these compounds can be bound to a sugar molecule, rendering them sensorially inactive (D'Incecco *et al*., 2004; Swiegers *et al*., 2005). The sugar molecule bound to these compounds usually constitutes a glucose molecule, which denotes that LAB with the ability to demonstrate *β*-glucosidase activity could release the volatile compounds to become odour-active, which would enable them to contribute to the sensory profile of the wine (Grimaldi *et al*., 2000; Boido *et al*., 2002; Barbagallo *et al*., 2004; D'Incecco *et al*., 2004; Matthews *et al*., 2004; Spano *et al*., 2005). According to literature, there are conflicting results regarding the absence or presence of enzyme activity in synthetic media/wine-like conditions, as well as under actual wine conditions (Guilloux-Benatier *et al*., 1993; McMahon *et al*., 1999; Grimaldi *et al*., 2000; Mansfield *et al*., 2002; Barbagallo *et al*., 2004). Six of the seven (86%) *L. plantarum* strains characterised in this study have the potential to release glycosidically bound flavour compounds, compared to the absence of this *β*-glucosidase gene in the seven *O. oeni* strains. In our results, which are in accordance with findings by Mtshali (2007), this characteristic seems to be more widespread amongst *L. plantarum* strains than *O. oeni*, although this is a trait than can be an advantageous addition to the selection criteria for MLF starter cultures. Although the *β*-glucosidase gene has been characterised in both *L. brevis* (Michlmayr *et al*., 2010) and *L. plantarum* (Spano *et al*., 2005), further characterisation is needed to better understand this liberation process and possible enzyme activity under wine conditions. Lactic acid bacteria are also able to metabolise various phenolic acids present in grape must and wine via PAD activity. These resulting aroma compounds can either impart negative sensorial qualities to the wine or add to the aroma complexity depending on the concentration, which highlights the importance of these genes and the possible contribution to the wine aroma profile (Cavin *et al*., 1993; Lonvaud-Funel, 1999; Swiegers *et al*., 2005).

On the other hand, protease activity in wine LAB have

not been fully investigated or characterised. There is a possibility that LAB protease activity can degrade proteins found in wine and could concomitantly produce amino acids and peptides that could alter the wine aroma profile. The peptidases, including cysteine aminopeptidase, proline iminopeptidase, methionine aminopeptidase and membrane alanine aminopeptidase can potentially contribute to the development of the flavour profile of wine, if enzymes are active under wine conditions, by releasing free amino acids which can serve as precursors for aroma compounds (Matos *et al*., 1998; Mistou & Gripon, 1998). The *L. plantarum* strains also displayed a more diverse peptidase enzyme profile and therefore have a greater chance in releasing amino acids which can serve as aroma compound precursors.

Citrate lyase plays an integral role in the production of the flavour compound diacetyl and this enzyme is responsible for the cleavage of citrate into oxaloacetate and acetate. This is a multi-enzyme complex, consisting of γ-, α- and *β*-subunits, all of these playing a role in citrate metabolism (Bekal *et al*., 1998). The enzyme α-acetolactate synthase also plays a role in the citric acid metabolism pathway responsible for the production of diacetyl, one of the most important aroma compounds associated with MLF. Our findings are in accordance of those generated by Mtshali *et al*. (2010) where it was also found that at least 70% of the *L. plantarum* strains were positive for one or more of the citrate lyase genes. Therefore *L. plantarum* strains in our study could potentially play a greater role in the citric acid metabolic pathway and concomitant diacetyl production.

Esterases that originate from wine LAB are responsible for both the biosynthesis and hydrolysis of esters (Matthews *et al*., 2004; Sumby *et al*., 2009; Brod *et al*., 2010). *Oenococcus oeni*, as well as species of *Lactobacillus* and *Pediococcus*, is able to hydrolyse esters and Matthews *et al*. (2006) found that significant esterase activity levels remained under wine-like conditions. This implies that esterase activity originating from LAB could potentially contribute to the ester profile. All of the LAB strains screened in our study were positive for the esterase gene, compared to the 70% of the *L. plantarum* strains screened by Mtshali *et al*. (2010). Although our results only confirm the presence or absence of the esterase-encoding gene it remains to be seen if these genes are expressed under wine-conditions and if they synthesise or hydrolyse esters. There is great variation between species and genera with regards to their esterase activity and from available literature, it seems as if *O. oeni* displays the greatest activity, compared to lactobacilli and pediococci (Matthews *et al*., 2006). Recently, Brod *et al*. (2010) characterised a novel esterase from *L. plantarum* and found substrate specificity towards *p*-nitrophenyl-linked substrates, similar to that of *O. oeni* (Matthews *et al*., 2006).

The additional screening for the presence of enzymes responsible for the formation of volatile sulphur compounds will also be indicative of the potential contribution of the various LAB strains to the wine aroma profile. These enzymes can either catabolise amino acids to produce volatile sulphur compounds like methanethiol and dimethyl disulphide or play a role in the biosynthesis of L-methionine or L-cysteine (Lee *et al*., 2007; Knoll *et al*., 2010). Based on the activity of these enzymes, LAB cultures have a profound effect on the production of volatile aroma compounds. Based on our results, it is possible that the *O. oeni* strains could make a slightly greater contribution to the production of volatile sulphur compounds. Despite these results confirming the presence of the gene, during the characterisation of this gene in two *O. oeni* strains by Knoll *et al*. (2010), it was found that *O. oeni* seems to play only a minor role in the production of volatile sulphur compounds due to the possible lack of enzyme activity under wine conditions.

The ability to degrade arginine and produce ethyl carbamate precursors have been found amongst both *O. oeni* and *L. plantarum* strains, although conflicting results in the literature would indicate that this ability is strain dependant. In addition, this characteristic also seems to be more widespread amongst *O. oeni* strains, compared to *L. plantarum* (Liu *et al*., 1994, 1995; Mira de Orduña *et al*., 2000, 2001; Spano *et al*., 2004; Uthurry *et al*., 2006; Araque *et al*., 2009; Romero *et al*., 2009). These findings in literature are supported by the finding in this study: six of the seven *O. oeni* strains were positive for arginine deiminase and carbamate kinase and all seven were positive for ornithine transcarbamylase. Contrary to this, none of the *L. plantarum* strains were positive for arginine deiminase, the first enzyme in the pathway that is responsible for the production of ethyl carbamate precursors.

Despite the results of the enzymatic screenings, it needs to be taken into consideration that these PCR reactions only confirm the presence/absence of the genes; it does not give any indication of the expression, if any, of these genes under winemaking conditions, nor is it indicative of the enzymatic activity.

The LAB isolates evaluated during the course of the small-scale fermentation seem to be compatible with the yeast strain *S. cerevisiae* WE372. No immediate or drastic decrease in cell counts was observed after inoculation for MLF, which indicates that metabolites and by-products produced by the wine yeast did not have a detrimental effect on the bacteria. Further investigation is required to elucidate the compatibility of the bacterial isolates with other wine yeast strains as well as the influence of different inoculation regimes on the yeast-bacteria interactions. Despite the fact that the *L. plantarum* strains took an average of 44 days to complete MLF, compared to nine days required by the *O. oeni* strains, this experiment served as an initial screening process to establish the ability of the isolates to tolerate the challenging wine environment.

At the completion of this study we were able to select three potential starter strains of *O. oeni* (S5, S6 and E53) and three *L. plantarum* strains (14.1, 56 and 107) that show potential for future use as malolactic starter cultures. These six strains were selected based on their malic acid degradation ability in wine, their ability to survive the wine environment, as well as their inability to act as major contributors to the volatile acidity concentration. As a result of the characterisation of the isolates, the genetic potential with regards to the production of aroma- and health-impacting compounds, are known.

In conclusion, this study showed, initially in synthetic medium and then in wine, that both *O. oeni* and *L. plantarum* have the potential to be utilised as MLF starter cultures when

used to inoculate after the completion of AF in high alcohol wines. *Lactobacillus plantarum* strains might have an added beneficial influence in that it has the genetic potential to influence the wine aroma profile to a larger extent than *O. oeni*, due to its cache of enzymes.

LITERATURE CITED

Araque, I., Gil, J., Carreté, R., Bordons, A. & Reguant, C., 2009. Detection of *arc* genes related with the ethyl carbamate precursors in wine lactic acid bacteria. J. Agric. Food Chem. 57, 1841-1847.

Arena, M.E. & Manca de Nadra, M.C., 2001. Biogenic amine production by *Lactobacillus*. J. App. Microbiol. 90*,* 158-162.

Arena, M.E., Manca de Nadra, M.C. & Muñoz, R., 2002. The arginine deiminase pathway in the wine lactic acid bacterium *Lactobacillus hilgardii* X_1B: structural and functional study of the *arcABC* genes. Gene 301, 61-66.

Barbagallo, R.N., Spagna, G., Palmeri, R. & Torriani, S., 2004. Assessment of *β*-glucosidase activity in selected wild strains of *Oenococcus oeni* for malolactic fermentation. Enzyme Microb. Tech. 34, 292-296.

Bekal, S., Diviès, C. & Prèvost, H., 1998. Citrate lyases of lactic acid bacteria. Lait, 78, 3-10.

Boido, E., Lloret, A., Medina, K., Carrau, F. & Dellacassa, E., 2002. Effect of *β*-glucosidase activity of *Oenococcus oeni* on the glycosylated flavor precursors of Tannat wine during malolactic fermentation. J. Agric. Food Chem. 50, 2344-2349.

Bou, M. & Krieger, S., 2004. Alcohol-tolerant malolactic strains for the maturation of wines with average or high pH (Pub. N°: WO/2004/111179; PCT/FR2004/001421).

Britz, T.J. & Tracey, R.P., 1990. The combination effect of pH, SO_2, ethanol and temperature on the growth of *Leuconostoc* oenos. J. App. Bacteriol. 68, 23-31.

Brod, F.C.A., Vernal, J., Bertoldo, J.B., Terenzi, H. & Arisi, A.C.M., 2010. Cloning, expression, purification, and characterization of a novel esterase from *Lactobacillus plantarum*. Mol. Biotechnol. 44, 242-249.

Campos, F.M., Couto, J.A. & Hogg, T.A., 2003. Influence of phenolic acids on growth and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii*. J. Appl. Microbiol. 94, 167-174.

Capozzi, V., Russo, P., Beneduce, L., Weidmann, S., Grieco, F., Guzzo, J. & Spano, G., 2010. Technological properties of *Oenococcus oeni* strains isolated from typical southern Italian wines. Lett. Appl. Microbiol. 50, 327-334.

Cavin, J.F., Andioc, V., Etievant, P.X. & Davies, C., 1993. Ability of wine lactic acid bacteria to metabolize phenol carboxylic acids. Am. J. Enol. Vitic. 44, 76-80.

Coton, M., Romano, A., Spano, G., Ziegler, K., Vetrana, C., Desmarais, C., Lonvaud-Funel, A., Lucas, P. & Coton, E., 2010. Occurrence of biogenic amine-forming lactic acid bacteria in wine and cider. Food Microbiol. 27, 1078-1085.

Davis, C.R., Wibowo, D., Fleet, G.H., & Lee, T.H., 1988. Properties of wine lactic acid bacteria: Their potential enological significance. Am. J. Enol. Vitic. 39, 137-142.

D'Incecco, N., Bartowsky, E., Kassara, S., Lante, A., Spettoli, P. & Henschke, P., 2004. Release of glycosidically bound flavour compounds of Chardonnay by *Oenococcus oeni* during malolactic fermentation. Food Microbiol. 21, 257-265.

Downing, L., 2003. Characterisation of biogenic amine-encoding genes in lactic acid bacteria isolated from South African wine. MSc. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Drici-Cachon, A., Guzzo, J., Cavin, F. & Diviès, C., 1996. Acid tolerance in *Leuconostoc oenos*. Isolation and characterisation of an acid resistant mutant. Appl. Microbiol. Biotechnol. 44, 785-789.

Du Toit, M., Engelbrecht, L., Lerm, E. & Krieger-Weber, S., 2010. *Lactobacillus*: the next generation of malolactic fermentation starter cultures-an overview. Food Bioprocess Technol. 4: 876-906.

Fugelsang, K.C. & Edwards, C.G., 1997 (2nd ed). Wine Microbiology: Practical Applications and Procedures. Springer, New York.

Fumi, M.D., Krieger-Weber, S., Déléris-Bou, M., Silva, A. & Du Toit, M., 2010. A new generation of malolactic starter cultures for high pH wines. Proceedings International IVIF Congress 2010, WB3 Microorganisms-Malolactic-Fermentation.

G-Alegría, E., López, I., Ruiz, J.I., Sáenz, J., Fernández, E., Zarazaga, M., Dizy, M., Torres, C. & Ruiz-Larrea, F., 2004. High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. FEMS Microbiol. Lett. 230, 53-61.

Grimaldi, A., McLean, H. & Jiranek, V., 2000. Identification and partial characterization of glycosidic activities of commercial strains of the lactic acid bacterium, *Oenococcus oeni*. Am. J. Enol. Vitic. 51, 362-369.

Guerrini, S., Mangani, S., Granchi, L. & Vincenzini, M., 2002. Biogenic amine production by *Oenococcus oeni*. Curr. Microbiol. 44, 374-378.

Guerzoni, M.E., Sinigaglia, M., Gardini, F., Ferruzzi, M. & Torriani, S., 1995. Effects of pH, temperature, ethanol, and malate concentration on *Lactobacillus plantarum* and *Leuconostoc oenos*: Modelling of the malolactic activity. Am. J. Enol. Vitic. 3, 368-374.

Guilloux-Benatier, M., Son, H.S., Bouhier, S. & Feuillat, M., 1993. Activite's enzymatiques: glycosidases et peptidase chez *Leuconostoc oenos* au cours de la croissance bacte'rienne. Influence des amcrolome'cules de levures. Vitis 32, 51-57.

Henick-Kling, T., 1993. Malolactic fermentation. In: Fleet, G.H. (ed.). Wine Microbiology and Biotechnology, Harwood Academic Publishers, Chur, Switzerland, pp. 289-326.

Jackson, R.S., 2008 (3rd ed). Wine Science: Principles and applications. Academic Press, California, USA. p. 394.

Knoll, C., Du Toit, M., Schnell, S., Rauhut, D. & Irmler, S., 2010. Cloning and characterization of a cystathionine β/γ-lyase from two *Oenococcus oeni* oenological strains. Appl. Microbiol. Biotechnol. 89, 1051-1060.

Landete, J.M., Ferrer, S. & Pardo, I., 2005. Which lactic acid bacteria are responsible for histamine production in wine? J. Appl. Microbiol. 99, 580-586.

Landete, J.M., Ferrer, S. & Pardo, I., 2007. Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. Food Control 18, 1569-1574.

Lee, W.-J., Banavara, D.S., Hughes, J.E., Christiansen, J.K., Steele, J.L., Broadbent, J.R. & Rankin, S.A., 2007. Role of cystathionine *β*-lyase in catabolism of amino acids to sulfur volatiles by genetic variants of *Lactobacillus helviticus* CNRZ 32. Appl. Environ. Microbiol. 73, 3034-3039.

Lerm, E., 2010. The selection and characterisation of lactic acid bacteria to be used as a mixed starter culture for malolactic fermentation. MSc. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Lerm, E., Engelbrecht, L. & Du Toit, M., 2010. Malolactic fermentation: the ABC's of MLF. S. Afr. J. Enol.Vitic. 31, 186-212.

Lewington, J., Greenaway, S.D. & Spillane, B.J., 1987. Rapid small scale preparation of bacterial genomic DNA suitable for cloning and hybridization analysis. Lett. Appl. Microbiol. 5, 51-53.

Liu, S.-Q., Pritchard, G.G., Hardman, M.J. & Pilone, G.J., 1994. Citrulline production and ethyl carbamate (urethane) precursor formation from arginine degradation by wine lactic acid bacteria *Leuconostoc oenos* and *Lactobacillus buchneri*. Am. J. Enol. Vitic. 45, 235-242.

Liu, S.-Q., Pritchard, G.G., Hardman, M.J. & Pilone, G.J., 1995. Occurrence of arginine deiminase pathway enzymes in arginine catabolism by wine lactic acid bacteria. Appl. Environ. Microbiol. 61, 310-316.

Lonvaud-Funel, A., 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. A. van Leeuw. 76, 317-331.

Lonvaud-Funel, A., 2001. Biogenic amines in wine: role of lactic acid bacteria. FEMS Microbiol. Lett. 199, 9-13.

Louw, L., Roux, K., Tredoux, A., Tomic, O., Naes, T., Nieuwoudt, H.H. & van Rensburg, P., 2009. Characterisation of selected South African young cultivar wines using FTMIR spectroscopy, gas chromatography and multivariate data analysis. J. Agric. Food Chem. 57, 2623-2632.

Manfroi, L., Silva, P.H.A., Rizzon, L.A., Sabaina, P.S. & Glória, M.B.A., 2009. Influence of alcoholic and malolactic starter cultures on bioactive amines in Merlot wine. Food Chem. 116, 208-213.

Mansfield, A.K., Zoecklein, B.W. & Whiton, R., 2002. Quantification of glycosidase activity in selected strains of *Brettanomyces bruxellensis* and *Oenococcus oeni*. Am. J. Enol. Vitic. 53, 303-307.

Marcobal, Á., De Las Rivas, B., Moreno-Arribas, M.V. & Muñoz, R., 2005. Multiplex-PCR method for the simultaneous detection of histamine-, tyramine-, and putrescine producing lactic acid bacteria in foods. J. Food Protect. 68, 874-878.

Matos, J., Nardi, M., Kumura, H. & Monnet, V., 1998. Genetic characterisation of *pepC*, which encodes an aminopeptidase P whose deficiency does not affect *Lactococcus lactis* growth in milk, unlike deficiency of the x-prolyl dipeptidyl aminopeptidase. Appl. Environ. Microbiol. 64, 4591-4595.

Matthews, A., Grimaldi, A., Walker, M., Bartowsky, E., Grbin, P. & Jiranek, V., 2004. Lactic acid bacteria as a potential source of enzymes for use in vinification. Appl. Environ. Microbiol. 70, 5715-5731.

Matthews, A.H., Grbin, P.R. & Jiranek, V., 2006. A survey of lactic acid bacteria for enzymes of interest to oenology. Aust. J. Grape Wine Res. 12, 235-244.

McMahon, H., Zoecklein, B.W., Fugelsang, K. & Jasinsky, Y., 1999. Quantification of glycosidase activities in selected yeasts and lactic acid bacteria. J. Ind. Microbiol. Biotechnol. 23, 198-203.

Michlmayr, H., Schümann, C., Barreira Braz da Silva, N.M., Kulbe, K.D. & Del Hierro, A.M., 2010. Isolation and basic characterisation of a *β*-glucosidase from a strain of *Lactobacillus brevis* isolated from a malolactic starter culture. J. Appl. Microbiol. 108, 550-559.

Mira de Orduña, R., Liu, S.-Q., Patchett, M.L. & Pilone, G.J., 2000. Ethyl carbamate precursor citrulline formation from arginine degradation by malolactic wine lactic acid bacteria. FEMS Microbiol. Lett. 183, 31-35.

Mira de Orduña, R., Liu, S.-Q., Patchett, M.L. & Pilone, G.J., 2001. Growth and arginine metabolism of the wine lactic acid bacteria Lactobacillus buchneri and *Oenococcus oeni* at different pH values and arginine concentrations. Appl. Environ. Microbiol. 67, 1657-1662.

Mistou, M.-Y. & Gripon, J.-C., 1998. Catalytic properties of the cysteine aminopeptidase pepC, a bacterial bleomycin hydrolase. Biochim. Biophys. Acta 1383, 63-70.

Moreno-Arribas, V., Torlois, S., Joyeux, A., Bertrand, A. & Lonvaud-Funel, A., 2000. Isolation, properties and behaviour of tyramine-producing lactic acid bacteria from wine. J. Appl. Microbiology, 88, 584-593.

Mtshali, P.S., 2007. Screening and characterisation of wine-related enzymes produced by wine-associated lactic acid bacteria. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Mtshali, P.S., 2010. Personal communication.

Mtshali, P.S., Divol, B.T., Van Rensburg, P. & Du Toit, M., 2010. Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines. J. Appl. Microbiol. 108, 1389–1397.

Pozo-Bayón, M.A., G-Alegría, E., Polo, M.C., Tenorio, C., Martín-Álvarez, P.J., Calvo de la Banda, M.T., Ruiz-Larrea, F. & Moreno Arribas, M.V., 2005. Wine volatile and amino acid composition after malolactic fermentation: Effect of *Oenococcus oeni* and *Lactobacillus plantarum* starter cultures. J. Agric. Food Chem. 53, 8729-8735.

Romero, S.V., Reguant, C., Bordons, A. & Masqué, M.C., 2009. Potential formation of ethyl carbamate in simulated wine inoculated with *Oenococcus oeni* and *Lactobacillus plantarum*. Int. J. Food Sc. Technol. 44, 1206-1213.

Rosi, I., Nannelli, F. & Giovani, G., 2009. Biogenic amine production by *Oenococcus oeni* during malolactic fermentation of wines obtained using different strains of *Saccharomyces cerevisiae*. Food Sc. Technol. 42, 525-530.

Ruiz, P., Izquierdo, P.M., Seseña, S. & Palop, M.L., 2010. Selection of autochthonous *Oenococcus oeni* strains according to their oenological properties and vinification results. Int. J. Food Microbiol. 137, 230-235.

Solieri, L., Genova, F., De Paola, M. & Giudici, P., 2010. Characterization and technological properties of *Oenococcus oeni* strains from wine spontaneous malolactic fermentations: a framework for selection of new starter cultures. J. Appl. Microbiol. 108, 285-298.

Spano, G., Chieppa, G., Beneduce, L. & Massa, S., 2004. Expression analysis of putative *arcA*, *arcB* and *arcC* genes partially cloned from *Lactobacillus plantarum* isolated from wine. J. Appl. Microbiol. 96, 185-193.

Spano, G., Rinaldi, A., Ugliano, M., Moio, L., Beneduce, L. & Massa, S., 2005. A *β*-glucosidase gene isolated from wine *Lactobacillus plantarum* is regulated by abiotic stresses. J. Appl. Microbiol. 98, 855-861.

Sumby, K.M., Matthews, A.H., Grbin, P.R. & Jiranek, V., 2009. Cloning and characterization of an intracellular esterase from the wine-associated lactic acid bacterium *Oenococcus oeni*. Appl. Environ. Microbiology, 75, 6729-6735.

Swiegers, J.H., Bartowsky, E.J., Henschke, P.A. & Pretorius, I.S., 2005. Yeast and bacterial modulation of wine aroma and flavour. Aust. J. Grape Wine Res. 11, 139-173.

Terrade, N. & Mira de Orduña, R., 2009. Determination of the essential nutrient requirements of wine-related bacteria from the genera *Oenococcus* and *Lactobacillus*. Int. J. Food Microbiol. 133, 8-13.

Ugliano, M., Genovese, A. & Moio, L., 2003. Hydrolysis of wine aroma precursors during malolactic fermentation with four commercial starter cultures of *Oenococcus oeni*. J. Agric. Food Chem. 51, 5073-5078.

Uthurry, C.A., Suárez Lepe, J.A., Lombardero, J. & Garcia Del Hierro, J.R., 2006. Ethyl carbamate production by selected yeasts and lactic acid bacteria in red wine. Food Chem. 94, 262-270.

Vivas, N., Lonvaud-Funel, A. & Glories, Y., 1997. Effect of phenolic acids and anthocyanins on growth, viability and malolactic activity of a lactic acid bacterium. Food Microbiol. 14, 291-300.

Wibowo, D., Eschenbruch, R., Davis, D.R., Fleet, G.H. & Lee, T.H., 1985. Occurrence and growth of lactic acid bacteria in wine: A review. Am. J. Enol. Vitic. 36, 302-313.