PICFPA	SAAPPA / SASPA / SAT	DFTS	Winetech
Canning Fruit Producers' Assoc. Submit to: Wiehahn Victor Tel: +27 (0)21 872 1501 inmaak@mweb.co.za	Fruitgro Science Submit to: Louise Liebenberg Tel: +27 (0)21 882 8470/1 louise@fruitgro.co.za	Dried Fruit Technical Services Submit to: Dappie Smit Tel: +27 (0)21 870 2900 dappies@dtd.co.za	Submit to: Jan Booysen Tel: +27 (0)21 807 3324 booysenj@winetech.co.za

			x

Indicate (X) client(s) to whom this final report is submitted.
Replace any of these with other relevant clients if required.

FINAL REPORT
FOR 2012

PROGRAMME & PROJECT LEADER INFORMATION

	Programme leader	Project leader
Title, initials, surname	Prof. Maret du Toit	Dr Benoit Divol
Present position	Associate-Professor	Researcher
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PROJECT INFORMATION

Project number	IWBT-B 08/09
Project title	Identification of important genes from lactic acid bacteria for wine production and evaluating the influence of physical and chemical wine parameters on the activity and expression of the genes
Project Keywords	Enzyme encoding genes; MLF; *Lactobacillus florum*;

Industry programme	**CFPA**	
	Deciduous	
	DFTS	
	Winetech	Microbiology committee
	Other	

Fruit kind(s)	Wine
Start date (dd/mm/yyyy)	01/01/2008
End date (dd/mm/yyyy)	31/12/2011

(Note: adjust footer – insert the project number no, researcher and research institution)

FINAL REPORT

(Completion of points 1-5 is compulsory)

1. Executive summary

Give an executive summary of the *total* project in no more than 250 words

Lactic acid bacteria possess a wide range of potential enzymes with interesting traits from an oenological point of view. In this study, LAB strains isolated from South African grape and wine samples were genetically screened for the presence of genes encoding enzymes of oenological relevance using gene-specific primers. We investigated the presence of genes coding for enzymes involved in proteolytic and amino acid catabolic pathways. PCR detection results showed that most strains possessed different combinations of enzyme-encoding genes. The sequencing of PCR fragments also allowed us to study homology patterns of nucleotide gene sequences between different wine LAB species. Phylogenetic analysis also showed different patterns of clustering amongst different species. The presence of genes in our tested strains therefore shows the genetic potential of wine LAB to influence wine aromatic profile. In addition, we also identified several strains as *Lactobacillus florum*. In this study, a total of 30 strains isolated from South African grape and wine samples were identified as *Lb. florum* through 16S ribosomal DNA sequence analysis and by PCR with species-specific primers that was developed. This is the first association of this species with grapes or wine. The study also yielded species specific primers that can be used in future to identify this microbe. Wines were made with *O. oeni* strains, although MLF was not completed in the Chardonnay, the optimization of RNA extraction directly from was optimized, which in future can be used for expression studies. qPCR reactions for the house-keeping gene and citrate lyase was optimized.

2. Problem identification and objectives

State the problem being addressed and the ultimate aim of the project.

It is well known that winemaking involves the interaction of different microbial species to ensure a good quality wine. Wine is produced by a primary fermentation, mainly conducted by *Saccharomyces cerevisiae*, and a secondary fermentation called malolactic fermentation (MLF) performed by lactic acid bacteria (LAB). *S. cerevisiae* has been well-studied and therefore the performance and impact of this yeast on wine quality is well-established. On the other-hand the performance of LAB in the wine matrix and their potential impact on the final product is not well-understood, especially there contribution to wine aroma.

The ultimate goal of this project is to generate knowledge on the potential contribution of LAB and MLF starter cultures on the final chemical profile of wine and ultimately the sensorial impact.

3. Workplan (materials & methods)

List trial sites, treatments, experimental layout and statistical detail, sampling detail, cold storage and examination stages and parameters.

Milestone 1: To identify important genes involved in wine aroma production and unwanted compounds of different wine-associated LAB with PCR

The genes that will be targeted in the milestones are beta-glycosidases, glucanases, phenolic acid decarboxylases, proteases, citrate lyase, biogenic amine decarboxylases and lipases.

Task 1: To design primers and screen wine LAB and MLF commercial starter cultures with PCR for the above-mentioned enzymes

Database sequences will be aligned for the total ORF of each gene and primers will be designed using software. Primers will be tested and the reaction optimised with a control strain from literature. Colony PCR will be used for screening.

Task 2: To sequence fragments obtained in task 1 from selected strains

PCR products will be sent for sequencing at the Central Analytical Facility, US. If necessary fragments will be isolated from the gel and cloned into a standard vector and sequenced with universal primers.

Task 3: Use bioinformatic tools to determine similarities between genes of different species

Strains with interesting combinations of enzymes will be selected to be evaluated in milestone 2 and 3.

Milestone 2: To evaluate the influence of selected physical and chemical factors on the expression and activity of the genes of selected LAB strains in the controlled standardized model juice systems by qRT-PCR

Task 1: Based on the sequences obtained in milestone 1, new primers will be designed in order to amplify a short portion of all the aforementioned genes by quantitative real-time PCR

Task 2: Evaluation of wine parameters on the expression of the genes

Strains will be inoculated into model juice and wine systems and at different stages the RNAs will be extracted from the frozen cells after the completion of MLFs. Reverse-transcription will be carried out on RNA samples after purification. The quantitative real-time PCRs will be performed on the cDNA obtained after reverse-transcription in order to quantify the expression of the different genes throughout the time (beginning, middle and end of MLF) and in the different tested conditions (pH 3.2 and 3.7, ethanol 12 and 16%, temperature 14°C and 22°C). Chemical analysis of substrate and product will be determined with chemical analytical methods.

Milestone 3: Evaluation of the most promising *O. oeni* strains under winemaking conditions and their impact on the wine and sensorial quality

Task 1: Small-scale winemaking with selected LAB strains

Both red (Cabernet Sauvignon, Pinotage, Shiraz) and white wines (Chardonnay) will be made according to standard winemaking practices. Control (only alcoholic fermentation) and spontaneous MLF will be included. Three different wine yeast starter cultures and the three best LAB strains will be evaluated in combinations to assess the chemical interactions.

Task 2: Chemical analysis of wines

Wines will be analysed with HPLC (biogenic amines and organic acids), GC-FID (volatile aroma compounds), GC-MS (diacetyl) and FT-IR after alcoholic fermentation and malolactic fermentation.

4. Results and discussion

State results obtained and list any benefits to the industry. Include a short discussion if applicable to your results.
This final discussion must cover ALL accumulated results from the start of the project, but please limit it to *essential* information.

Milestone	**Achievement**
1. Screening of *Lactobacillus* strains for enzyme encoding genes	A total of 120 wine lactobacilli isolates belonging to *Lactobacillus plantarum*, *Lactobacillus hilgardii*,

	Lactobacillus brevis, *Lactobacillus pentosus*, *Lactobacillus paracasei*, *Lactobacillus sakei* and *Lactobacillus paraplantarum* were genetically screened for enzyme-encoding genes using PCR with primers specific for β-glucosidase, protease, esterase, citrate lyase and phenolic acid decarboxylase. The results of PCR-screening showed that the *Lactobacillus* strains possessed different combinations of enzymes and some strains did not possess any of the enzymes tested. Confirmation analysis with gene sequencing also showed high similarity of genes with those available in GenBank database.
2. Identification of *Lactobacillus florum*	30 strains previously isolated from South African grape and wine samples were identified performing BLAST and phylogenetic analyses of 16S rDNA gene sequences, which indicated that the isolates belonged to *Lactobacillus florum*. In this work, we also designed a discriminative species-specific primer FLOR targeting the 16S rDNA gene of *Lb. florum*. PCR detection results revealed that all *Lb. florum* strains possessed the majority of the tested genes.
3. Detection of enzyme encoding genes for wine *Leuconostoc mesenteroides*	Fifteen isolates of lactic acid bacteria originating from South African grape and wine samples were identified as *Leuconostoc mesenteroides* subsp. *mesenteroides* through the taxonomic analysis of their 16S rDNA gene sequences. From the PCR detection results, the *estA*, *prtP*, *alsD*, *alsS*, *metK*, *metC* and *metB* genes were present in all the strains tested. The *bgl* and *gshR* genes encoding β-glucosidase and glutathione reductase, respectively, were not detected in some strains. None of the tested strains possessed the genes encoding phenolic acid decarboxylase (*padA*), citrate permease (*citP*), citrate lyase (*citD*, *citE* and *citF*) and arginine deiminase pathway enzymes (*arcA*, *arcB* and *arcC*). The verification of PCR-generated fragments was performed by sequencing. GenBank database was used to search for homologous DNA sequences. The phylogenetic analyses revealed that there are genetic heterogeneities between strains of *Leuc. mesenteroides* species.
4. Peptide and amino acid utilization genes in wine LAB	104 strains of lactic acid bacteria were tested for the presence of genes encoding enzymes related to peptide and amino acid utilization in wine. Primers for

	PCR amplifications were designed from conserved regions of the genes. From the PCR detection results, it was observed that the genes tested for were distributed across different species of lactobacilli and pediococci investigated. However, some strains of *Pediococcus* did not possess certain enzyme-encoding genes, such as *pepO*, *pepT*, *metK* and *gshR*. In addition, *pepX* and *metB*/*metC* genes were not detected in any of the *Pediococcus* strains tested. *Lactobacillus plantarum* IWBT B349 strain was selected for gene sequence verification. From the comparative sequence analysis, it was observed that nucleotide gene sequences of this strain are highly identical to those of other *L. plantarum* strains in GenBank database. The results presented in this study provide an indication that lactobacilli and pediococci strains of wine origin have the genetic potential to degrade peptides and sulphur-containing amino acids during vinification.
5. Wines made with selected *O. oeni* strains and optimization of RNA extraction from wine	A protocol to extract total bacterial RNA from wine, based on the acid phenol extraction protocol was optimized. The improved protocol includes numerous washes of the cell pellet using PVP. Quantitative real-time PCR (qRT-PCR) was also optimized for the detection and quantification of the expression of the 16S (used as housekeeping genes), *mleA* and *citE*. After DNase treatment and reverse-transcription, qRT-PCR gave very good results with regards to standard curves: excellent linearity with regression coefficient >0.99) and PCR efficiencies equal to 100% for all three genes. Chardonnay grape juice was inoculated with *Oenococcus oeni* IWBT B030 or IWBT B040 using co-inoculation or sequential inoculation. Unfortunately, these assays did not allow us to follow gene expression during malolactic fermentation, as in all cases, MLF got stuck, with bacterial populations decreasing quickly after inoculation. Because of time constraints, the fermentations could not be repeated. Nevertheless, the methods have now been fully optimized and the experiment should be repeated in order to generate insightful results.

5. Accumulated outputs

List ALL the outputs from the start of the project.
The year of each output must also be indicated.

Technology development, products and patents

Indicate the commercial potential of this project (intellectual property rights or a commercial product(s)).

- PCR primers for wine aroma enzymes
- PCR method for each enzyme primer pair
- Gene sequences of natural South African LAB wine isolates
- Identification primers for *L. florum*
- Wine aroma potential of *O. oeni* isolates

Human resources development/training

Indicate the number and level (e.g. MSc, PhD, post doc) of students/support personnel that were trained as well as their cost to industry through this project. Add in more lines if necessary.

	Student level (BSc, MSc, PhD, Post doc)	Cost to project (R)
1.	Senzo Mtshali - PhD	37,500
2.	Talitha Greyling - TA	125,000
3.	Camille Dwoinikoff – Intern from France	2,697
4.		
5.		

Publications (popular, press releases, semi-scientific, scientific)

1. Mtshali, P.S., B.T. Divol, P. van Rensburg & M. du Toit. **2010**. Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines. *J Appl. Microbiol.* 108: 1389–1397.
2. Mtshali, P.S., Divol B. & M. du Toit. **2012**. Identification and characterization of *Lactobacillus florum* strains isolated from South African grape and wine samples. *Int. J. Food Microbiol.* 153: 106-113.
3. Mtshali, P.S., Divol B. & M. du Toit. **2012**. PCR detection of enzyme-encoding genes in *Leuconostoc mesenteroides* strains of wine origin. *W. J. Microbiol. Biotechnol.* **28**: 1443-1449.
4. Mtshali, P.S., Divol B. & M. du Toit. **2012**. Evaluating *Lactobacillus* and *Pediococcus* strains for enzyme-encoding genes related to peptide and amino acid utilization in wine. *Ann. Microbiol.* DOI: 10.1007/s13213-012-0466-z.

Presentations/papers delivered

1. **Du Toit, M.**, S. Mtshali, C. Knoll & B. Divol. **2008**. Genetic screening of wine lactic acid bacteria for enzyme encoding genes important in winemaking. Ninth Symposium on Lactic acid and bacteria, Egmond aan Zee, The Netherlands.
2. **Mtshali, P.S.**, B. Divol & M. du Toit. **2008**. Molecular screening of wine lactic acid bacteria enzymes using gene-specific primers. Thirty First Conference of the South African Society for Enology and Viticulture, Somerset West, South Africa.
3. **Mtshali, P.S.**, B.T. Divol & M. du Toit. **2009**. Genetic characterisation of *Lactobacillus lindneri* strains isolated from South African grapes and wine. 4th International SASEV Conference on Enology & Viticulture - BEYOND 2010, Cape Town, South Africa.
4. **Mtshali, P.S.**, B.T. Divol & M. du Toit. **2009**. Comparative analysis of genes coding for enzymes of oenological relevance in wine lactic acid bacteria. 4th International SASEV Conference on Enology & Viticulture - BEYOND 2010, Cape Town, South Africa.
5. **Du Toit, M.**, S. Malherbe, E. Lerm, C. Knoll, L. Engelbrecht & P.S. Mtshali. **2009**. Impact of lactic acid bacteria on wine aroma. Bio2Biz and South African Society for Microbiology, Durban, South Africa.

6. **Du Toit, M.**, C. Knoll, S. Malherbe, E. Lerm, L. Engelbrecht, J. Carstens, P.S. Mtshali & D. Rauhut. **2010**. Impact of lactic acid bacteria on wine aroma. International Intervitis Interfructa Congress, Stuttgart, Germany.
7. Mtshali, P.S., **B. Divol** & M. du Toit. **2010**. An investigation into lactic acid bacterial enzyme-encoding genes related to wine aroma formation. The 14th Australian Wine Industry Technical Conference, Adelaide, Australia.
8. Mtshali, P.S., **B. Divol**, & M. du Toit. **2010**. Identification and enzyme profiling of *Lactobacillus lindneri* strains isolated from South African grape and wine samples. The 14th Australian Wine Industry Technical Conference, Adelaide, Australia.
9. **Mtshali, P.S.**, B. Divol & M. du Toit. **2010**. Molecular screening of wine lactic acid bacteria enzymes using gene-specific primers. Cape Biotechnology Forum 2010, Somerset West, South Africa.
10. Du Toit, M. **2011**. Genetic screening for wine-related enzymes within wine lactic acid bacteria and influence on the wine aroma profile. University of Torino, Piedmont, Italy.
11. Du Toit, M. **2011**. Genetic screening for wine-related enzymes within wine lactic acid bacteria and influence on the wine aroma profile. University of Udine, Friuli, Italy.
12. Du Toit, M. **2011**. How does MLF impact on wine aroma? Lallemand-IWBT MLF workshop, Stellenbosch University, Stellenbosch.

4. Total cost summary of project

	Year	CFPA	Deciduous	DFTS	Winetech	THRIP	Other	TOTAL
Total cost in real terms for year 1	2008				R 250 000	R 118 750		**R 368 750**
Total cost in real terms for year 2	2009				R 300 000	R 150 000		**R 450 000**
Total cost in real terms for year 3	2010				R 180 000	R 69 660		**R 249 660**
Total cost in real terms for year 4	2011				R 150 000	R 75 000		**R 225 000**
Total cost in real terms for year 5								
TOTAL					**R 880 000**	**R 413 410**		**R 1 293 410**