LACTIC ACID BACTERIA: FRIEND OR FOE?

Prof Maret du Toit
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Prof Maret du Toit
Department of Viticulture and Oenology
Faculty of AgriSciences
Stellenbosch University

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ABOUT THE AUTHOR

Maret du Toit was born in Cape Town and schooled in Durbanville, South Africa. After school she pursued her studies at Stellenbosch University, enrolling for a BSc degree in 1986 with microbiology and genetics as majors. She enrolled for a BSc Honours in Microbiology in 1989 and started her career in research with a project focusing on the taxonomy of *Lactobacillus* spp. This industrially important group of lactic acid bacteria (LAB) fascinated her and inspired her to learn more about who they were and what their role in different industrially important products was. From 1990 to 1992 she pursued her MSc in Microbiology under the supervision of Prof Leon Dicks with a thesis entitled "Taxonomy of the subgenera *Thermobacterium* and *Streptobacterium* (Groups I and II) of the genus *Lactobacillus*". In 1992 she enrolled for her PhD with a dissertation entitled "Isolation, identification and characterisation of lactic acid bacteria with potential probiotic characteristics" with Prof Dicks. The project was in collaboration with Prof Wilhelm Holzapfel from the Federal Research Centre for Nutrition, Institute of Hygiene and Toxicology, Karlsruhe, Germany were she spent 29 months in his laboratory to execute experiments on the effect of selected probiotic strains *in vivo* on minipigs.

Prof du Toit was recruited in 1998 as postdoctoral fellow to the Institute for Wine Biotechnology to establish and drive a research group focusing on LAB associated with winemaking and their role in and contribution to wine quality. She has been permanently appointed as academic since 2001 and was promoted to professor in January 2014. She has graduated seven PhD and 34 MSc students. She has published 63 peer-reviewed papers, four chapters in books, many popular articles and more than 200 conference proceedings, both internationally and nationally.

She was President of the South African Society for Enology and Viticulture for three years. She is a member of the editorial boards of three journals and a regular reviewer of more than 20 accredited scientific journals. She has chaired the organising committees of several international conferences and also co-organised several scientific symposia, workshops and research seminars. She is involved on different levels in committees in the South African wine industry. She is currently the Head of the Department of Viticulture and Oenology and is also involved in the management of the newly established Institute for Grape and Wine Sciences.

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INTRODUCTION

My having ended up in science should not be surprising to anybody as my favourite questions as a child were (and probably still are today if you ask my students), Why?, What? and How? These are the key questions that you need to answer as researcher in any scientific project. For lactic acid bacteria (LAB) then, you might ask, Who are they?, What do they do? or Why are they important?

Who are the LAB? They belong to the phylum *Furmicutes* and in Class I of *Bacilli*. The most important group is the *Lactobacillales* with six families and 40 genera. They are Gram-positive, non-sporeforming cocci, coccobacilli or bacilli and are catalase negative (Ludwig et al. 2009). From the 1960s to the 1990s, the taxonomy of LAB has been based mainly on phenotypic and genotypic methods and then it shifted to phylogenetic sequence analysis, which showed the misclassification of many species where the isolates identified were just isolated from different environments. Today comparative whole genome sequencing is becoming increasingly popular in the classification of LAB and offers exciting possibilities for the future of LAB identification and characterisation. This technology has shown that in some species there are only 40–50% genome similarities among strains (Vandamme et al. 2014; Wassenaar and Lukjancenko 2014). LAB are fastidious and have a complex nutritional need for growth, including carbohydrates, amino acids, vitamins and minerals. Some of the wine LAB also need a tomato juice factor (Lerm et al. 2010).

The enormous genome diversity of LAB correlates with the phenotypic and genotypic diversity seen among strains from the same species from different ecosystems. This diversity is indicated by the different habitats isolated from and the prevailing conditions, such as low pH (wine, vinegar), temperatures higher than 50 °C (cheese), high levels of sorbic acid (juices), hops resistance (beer), ethanol (wine, sake) and the human gut with high levels of bile salts (Holzapfel and Wood 2014). The ability to survive in these conditions is strain dependent and is determined genetically. With the explosion of LAB genomes being sequenced and comparative functional genomic tools being used to better understand the biodiversity of and tolerance to specific environments of specific strains within a species is used today for selection of improved industrial strains with specific traits.

What do LAB do? They ferment! Glucose fermentation divides LAB into two groups, namely homolactic or heterolactic. The homolactics produce two molecules of lactic acid and two molecules of adenosine triphosphate (ATP). Heterolactics produce more than just lactic acid from glucose metabolism with the addition of ethanol or acetic acid and CO_2 and one molecule of ATP. Many of the raw materials that LAB fermentations occur in also contain organic acids that are important for fermentation as they contribute to the energy metabolism of several LAB. The two most important organic acids are malic acid and citric acid, and they are very important in wine and dairy production, for example (Endo and Dicks 2014).

Why are LAB important? As Holzapfel and Wood (2014) state in *Lactic acid bacteria – biodiversity and taxonomy*, LAB can be regarded as pioneers in early published microbiological/bacterial studies that were driven by problems experienced in the food and fermentation industries. Next to yeasts, LAB are the industrially most important group of microbes used in fermentation. LAB paved the way for the inoculation of specific strains with important characteristics to obtain a product with desired traits. The first industrial starter was a lactic acid bacterial strain applied in 1890 to cheese production. Today we know that LAB play a crucial role worldwide in the production of food and beverages and in ensuring food security through the production of several bioprotective compounds. LAB are essential in the production of dairy products, meats and vegetables and play an important secondary role in the fermentation of silage, cocoa, coffee, sourdough, wine and many indigenous African fermented products. LAB are also important in human and animal health, such as pre- and probiotics and feed supplements. LAB are furthermore used as biotechnological agents for the production of macromolecules, enzymes and metabolites (Giraffa 2014). LAB primarily preserve food and beverages by producing lactic acid, but they are also used to provide variety in the food consumed by altering

the aroma, flavour, texture and appearance of the raw commodities in a favourable way.

Unfortunately, in many fermented products LAB are also regarded as the main spoilage agent. LAB can influence the texture of the product (polysaccharides = sliminess) and produce off-flavours (putrescine = rotten) and compounds that individuals are allergic to (biogenic amines = red cheeks). Therefore, it is important to manage the natural flora associated with the raw material to ensure that the spoilage LAB do not dominate and have a negative impact on the final product (Du Toit and Pretorius 2000).

To discuss whether LAB should be regarded as friend or foe, their role in winemaking will be used as model.

WHO ARE THE LACTIC ACID BACTERIA ASSOCIATED WITH WINEMAKING?

Winemaking consists of two fermentation processes. Alcoholic fermentation (AF) is the primary fermentation process in wine, carried out by yeast, mainly the more alcohol-tolerant *Saccharomyces cerevisiae* that convert sugar to ethanol and CO_2. Malolactic fermentation (MLF) is a secondary fermentation process conducted by LAB; it is a decarboxylation process whereby L-malic acid is converted to L-lactic acid with the production of CO_2 (Figure 1). The three main reasons for conducting MLF in wine are to deacidify the wine, to improve the microbial stability of the wine by removing malic acid (malate) as a possible carbon source and to modify wine aroma. Malolactic fermentation can modify wine aroma via the production or modification of flavour-active compounds (Malherbe et al. 2012; Michlmayr et al. 2012; Swiegers et al. 2005).

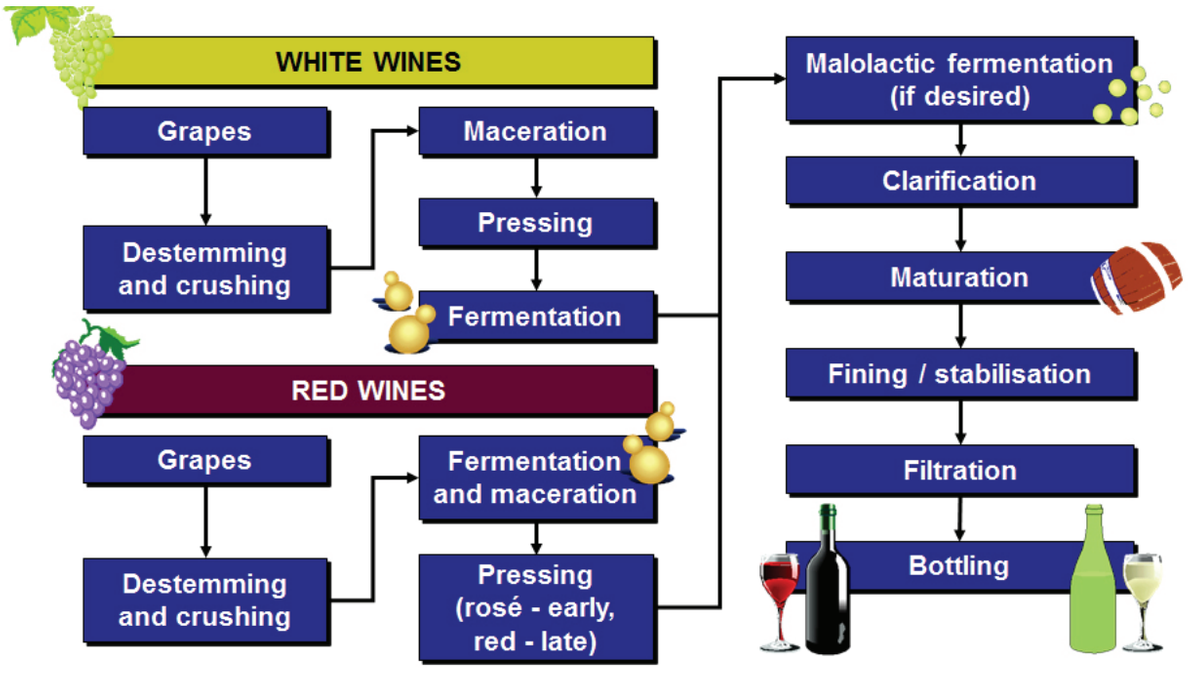

Figure 1: Diagram to show the basic steps in red and white winemaking

The main LAB associated with wine belong to the genera *Oenococcus (O.)*, *Lactobacillus (L.)*, *Pediococcus (P.)* and *Leuconostoc (Lc.)*. A large culture collection of South African LAB isolates has been established over the last two decades at the Institute for Wine Biotechnology. The isolations were done from the vineyard to bottled products that were spoiled. We have shown that the major LAB species found internationally in winemaking are also found locally. The major species associated with winemaking in South Africa are *O. oeni, L. plantarum, L. hilgardii, L. brevis* and *L. casei/paracasei* (Krieling 2003). Mtshali et al. (2012) for the first time associated wine-isolated lactobacilli with *L. florum* using rRNA sequencing.

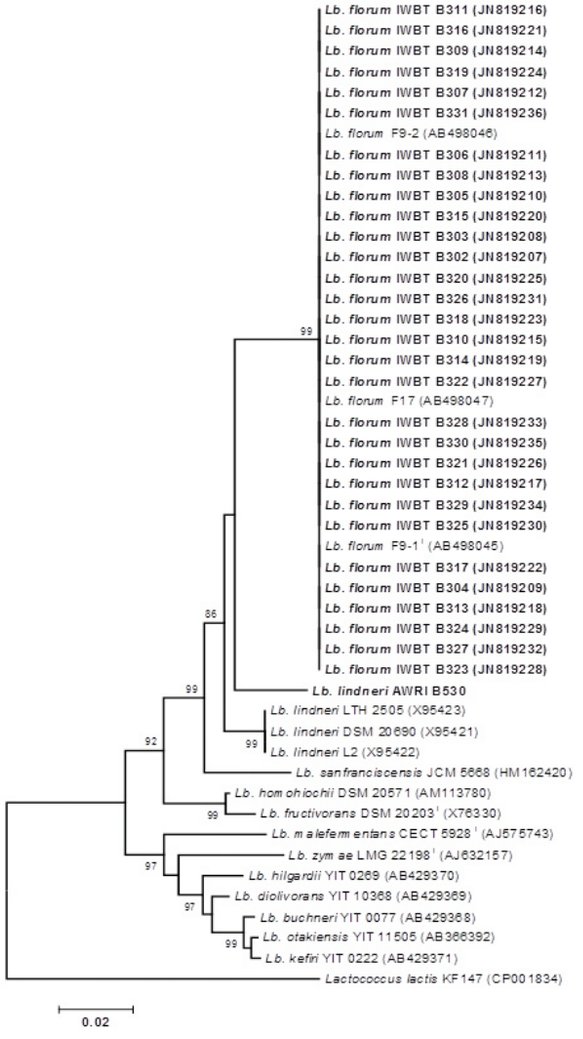

Figure 2: Phylogenetic tree of the South African wine isolates identified as L. florum (Mtshali et al. 2012)

The closest species was *L. lindneri*, which had been found on Australian grapes (Figure 2).

Of the four LAB genera found in wine, *O. oeni* is the best adapted to overcome high ethanol levels, low pH conditions and fermentation temperatures as well as SO_2, all of which make wine a harsh environment. This explains the predominant use of *O. oeni* as MLF starter cultures today; however, *L. plantarum* has proved its resilience and is therefore now also included in MLF starter cultures, especially for high-pH and low-sulphur wines for co-inoculation with yeast or before AF (Du Toit et al. 2011; Lerm et al. 2010).

WHAT WINE CHARACTERISTICS DO LACTIC ACID BACTERIA POSSESS?

The characteristic that is the most important from a winemaking perspective is the ability to degrade malic acid to lactic acid as this is the base of MLF. It has been shown in different studies that all species of the four main wine LAB have the malolactic enzyme gene (*mle*). It has been shown using *in silico* sequence analysis that the *mle* gene of the wine LAB species is different, and this might be one of the reasons why the strains break down malic acid differently (Figure 3) (Miller et al. 2011).

There are various enzymes that LAB possess that can play a role in the wine aroma profile and the quality of wines undergoing MLF. Some of the aroma-related enzymes that are important include *β*-glucosidase, phenolic acid decarboxylase, esterase, protease, peptidases, citric acid metabolism and production of volatile sulphur compounds. Other enzymes of interest are those associated with spoilage compounds, such as biogenic amines, ethyl carbamate, acrolein, ropiness and mousiness. It is important to select commercial strains that do not have these characteristics to minimise the risk of spoilage. Looking at the genome sequences of *L. plantarum* and *O. oeni*, one can see that they differ tremendously and therefore the conditions in which they will function as well as their impact on wine aroma will differ (Figure 3) (Mtshali 2011).

Enzyme:	*L. plantarum*	*O. oeni*	Significance:
Malolactic enzyme	+	+	Covert malic acid to lactic acid
β-D-glucosidase	+	-	Release of glycosidically bound aroma compounds
Phenolic acid decarboxylase	+	-	Metabolism of phenolic acids
Proline iminopeptidase	+	-	Release of free amino acids as aroma precursors
Citrate lyase α-subunit	+	-	Diacetyl production
Arginine deiminase	-	+	Ethyl carbamate production
Esterase	+	+	Synthesis or hydrolysis of esters

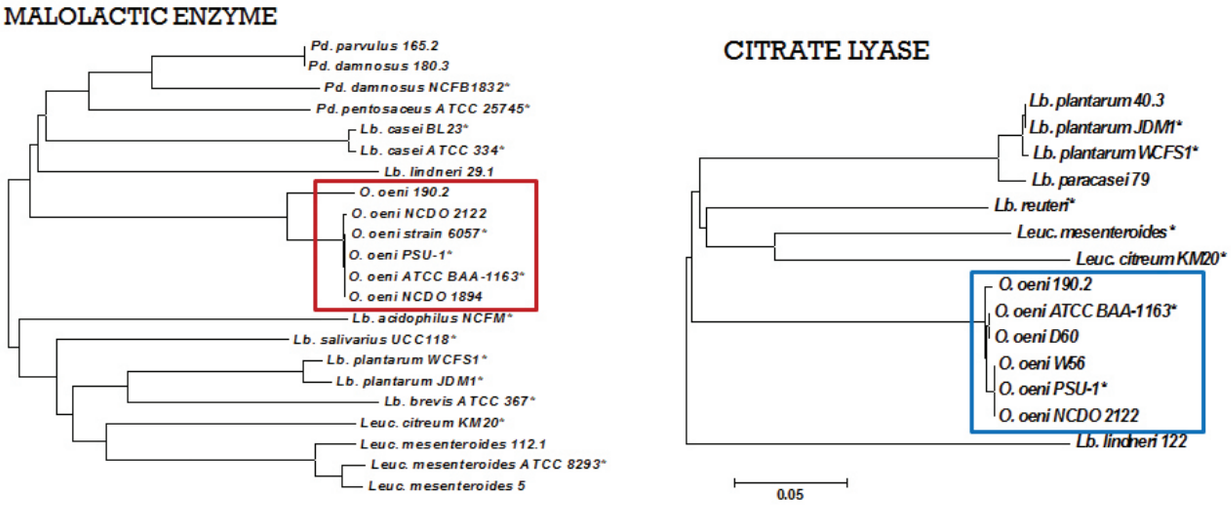

Figure 3: Differences between L. plantarum and O. oeni regarding the possession of some important genes. The phylogenetic relationship of the malolactic gene is on the left and the citrate lyase gene on the right.

The other characteristic that makes LAB very attractive is the ability to produce antimicrobial proteins or bacteriocins. Several plantaricin-producing *L. plantarum* strains of oenological origin that could be used to suppress the growth of natural LAB present during winemaking were previously identified. Knoll et al. (2008) showed that wine isolates from *L. paracasei, L. hilgardii* and *L. plantarum* produced bacteriocins. This was the first report on *L. hilgardii* producing an antimicrobial protein (Figure 4). Miller (2010) screened plantaricin-producing *L. plantarum* strains for the structural, transport and regulatory genes by polymerase chain reaction (PCR). Several plantaricin genes were identified in 20 *L. plantarum* strains, and two structural genes (*pln*EF and *pln*N), a transporter gene (*pln*G) and a histidine protein kinase gene (*pln*B) were sequenced and found to be highly conserved among the 20 strains. Plantaricin gene expression studies using qPCR showed that the structural genes *pln*JK and *pln*EF and the bacteriocin transporter gene *pln*G were expressed to varying degrees, depending on the fermentation conditions.

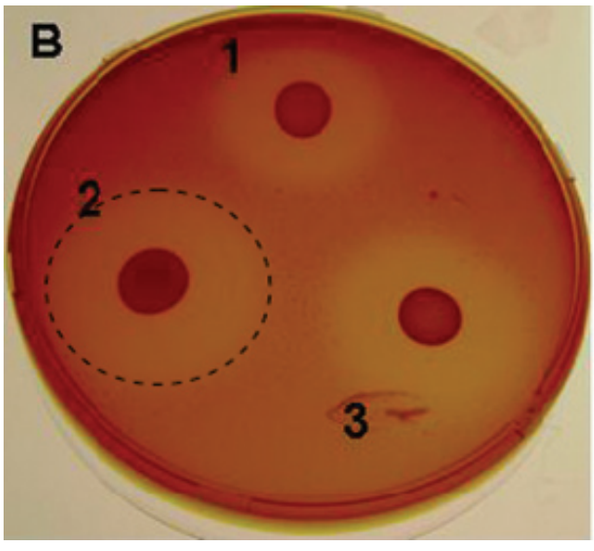

Figure 4: Bacteriocin-producing LAB show zones of inhibition against a sensitive LAB strain

MALOLACTIC FERMENTATION

Malolactic fermentation is conducted in most red and some white and sparkling wines. In the complex, harsh wine environment containing different microorganisms that compete for survival, many factors can influence LAB growth and therefore successful completion of MLF. These factors include high ethanol concentration (can exceed 15% v/v), low pH (can be less than 3.2), low temperature and SO_2 concentration (can be more than 50 mg/L), lysozymes, phenolic compounds, medium-chain fatty acids, yeast-bacteria interactions and nutrient availability (Alexandre et al. 2004; Bartowsky and Borneman 2011; Lerm et al. 2010).

Currently two main inoculation strategies are used by winemakers, each with its own challenges when selecting strains. The traditional MLF inoculation scenario is to inoculate after AF, when the greatest pressure on the strains is a high alcohol content, a low nutrient status and a high pH with a higher natural LAB population participating in MLF (Du Toit et al. 2011; Lerm et al. 2010). In this scenario, *O. oeni* strains still fare the best, but *L. plantarum* and *L. hilgardii* have shown that selected strains can perform MLF just as well (Du Toit et al. 2011; Lerm et al. 2011). The second strategy is co-inoculation or inoculating in the juice, especially in high-alcohol wines as it is the major factor that is responsible for problematic MLF. Co-inoculation where alcohol is not the biggest factor will definitely open the door for other species being used for MLF. Therefore, many of the studies that have looked at the natural isolates of a country or a region or a cultivar might deliver novel strains with interesting characteristics to be used for MLF in future. Spain has produced many studies that have looked at the natural isolates of specific regions or cultivars and therefore have generated a large collection of potential MLF strains, as seen in the studies by López et al. (2011) and Ruiz et al. (2010), for example.

MALOLACTIC FERMENTATION AND WINE AROMA

The production of flavour and aroma compounds is a result of the metabolism of grape constituents, such as sugars, amino acids and organic acids and/or the modification of grape- and yeast-derived aroma compounds (Bartowsky and Borneman 2011; Swiegers et al. 2005). The groups of compounds that are mostly impacted by MLF are carbonyl compounds, esters, higher alcohols, aldehydes, sulphur- and nitrogen-containing compounds, volatile phenols and volatile fatty acids (Lerm et al. 2011). The changes in aroma and flavour profiles during MLF are dependent on the bacteria strain responsible for MLF, the grape cultivar, winemaking practices, yeast-bacteria interactions, time of inoculation and enzymatic activity of the MLF strain.

Influence of yeast strain

It was shown that the selection of wine yeast strain would impact on wine aroma compounds and levels produced by LAB strains during MLF. Esters responsible for fruity characters differed significantly depending on the yeast strain used. MLF has the biggest impact on ethyl lactate and diethyl succinate and enhances the levels of other esters produced by the yeast. Therefore, the selection of yeast strain with MLF is important as it will impact the final aroma and style of the wine (Schöltz 2013).

Influence of lactic acid bacteria strain

Malherbe et al. (2012) evaluated the influence of different MLF *O. oeni* starter cultures on the volatile aroma composition using Pinotage and Shiraz. Changes were observed in ester concentrations after the completion of MLF. Synthesis and hydrolysis of esters during MLF were evident. Ethyl lactate, diethyl succinate, ethyl octanoate, ethyl 2-methylpropanoate and ethyl propionate concentrations were increased during MLF compared with the control wine for all four *O. oeni* strains. Increases in the concentrations of most of the higher alcohols were observed in MLF wines. Isoamyl alcohol, isobutanol, 2-phenylethanol, propanol, butanol, hexanol, 3-methyl-1-pentanol and 3-ethoxy-1-propanol concentrations were significantly increased by MLF, which indicates the potential contribution to specific characteristics in wine (Figure 5). Malherbe et al. (2013) showed that the changes in wine aroma compounds or ratios carried through to the sensory perception of consumers. MLF-treated wines compared to the control had more of a buttery character and fruitier aromas.

Apart from producing different ester ratios, the biggest impact on using *L. plantarum* versus *O. oeni* is

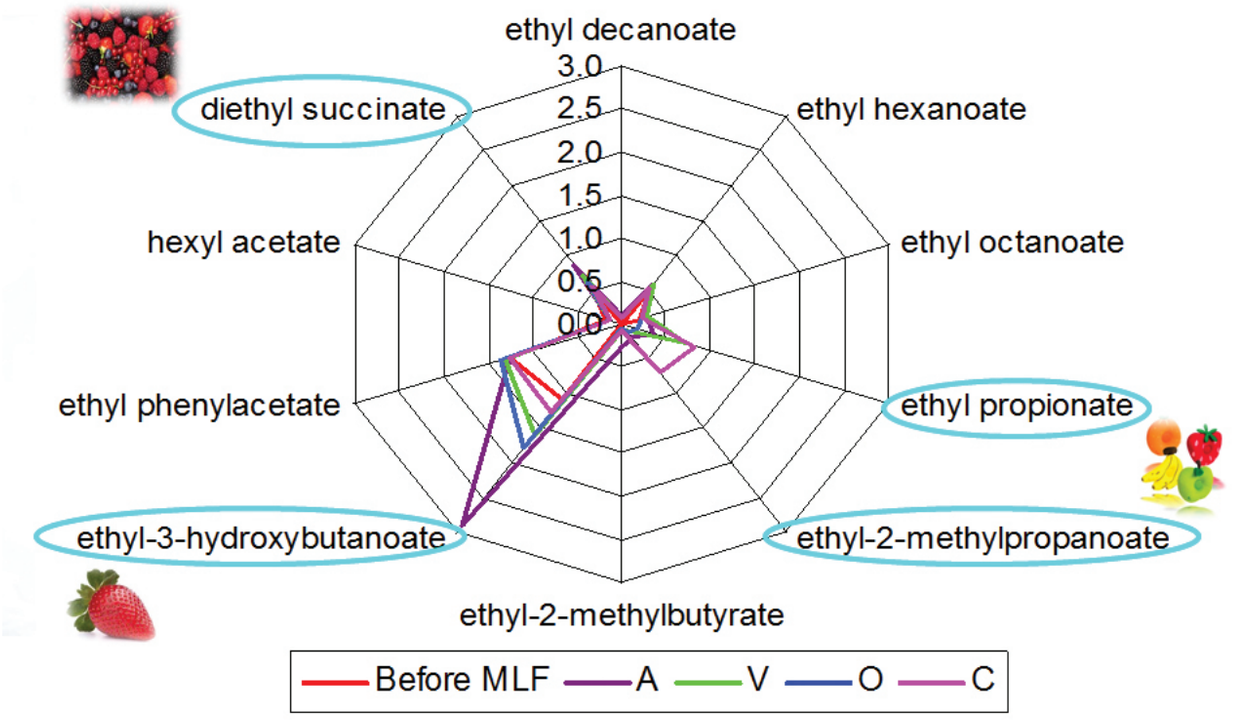

Figure 5: Graph of the ester contribution imparted by four different MLF starter cultures during MLF in Pinotage 2008 (Adapted from Malherbe et al. 2012)

related to the release of monoterpenes due to ß-glycosidase activity (Figure 6) (Lerm et al. 2012).

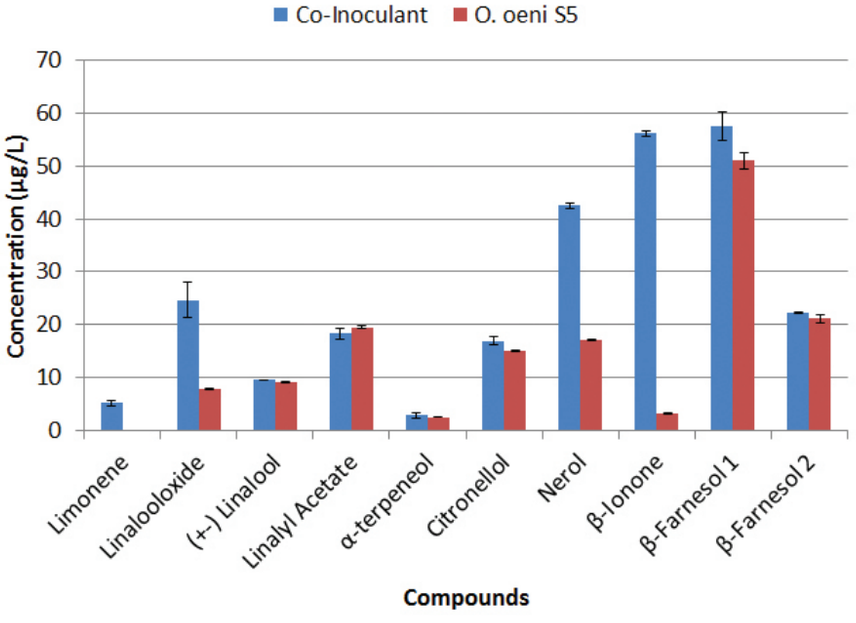

Figure 6: Comparison of the monoterpene production (excluding geraniol) of the mixed culture containing O. oeni and L. plantarum, the individual O. oeni strain from the mix and two O. oeni commercial cultures during co-inoculation in Shiraz in 2011 (Lerm et al. 2012)

Impact of malolactic fermentation inoculation scenario

Knoll et al. (2012) evaluated four different MLF inoculation scenarios: co-inoculation, 40% AF, 60% AF and sequential inoculation using two different *O. oeni* starter cultures. The results showed that the different inoculation scenarios were driven by different esters and resulted in different wine aroma profiles. Co-inoculated wines showed higher concentrations of ethyl and acetate esters, including acetic acid phenylethylester, acetic acid 3-methylbutylester, butyric acid ethylester, lactic acid ethylester and succinic acid diethylester when compared to sequential inoculation. The strain differences were more profound in co-inoculation and 40% AF where there was a clear separation between the two strains and the esters that they produced (Figure 7).

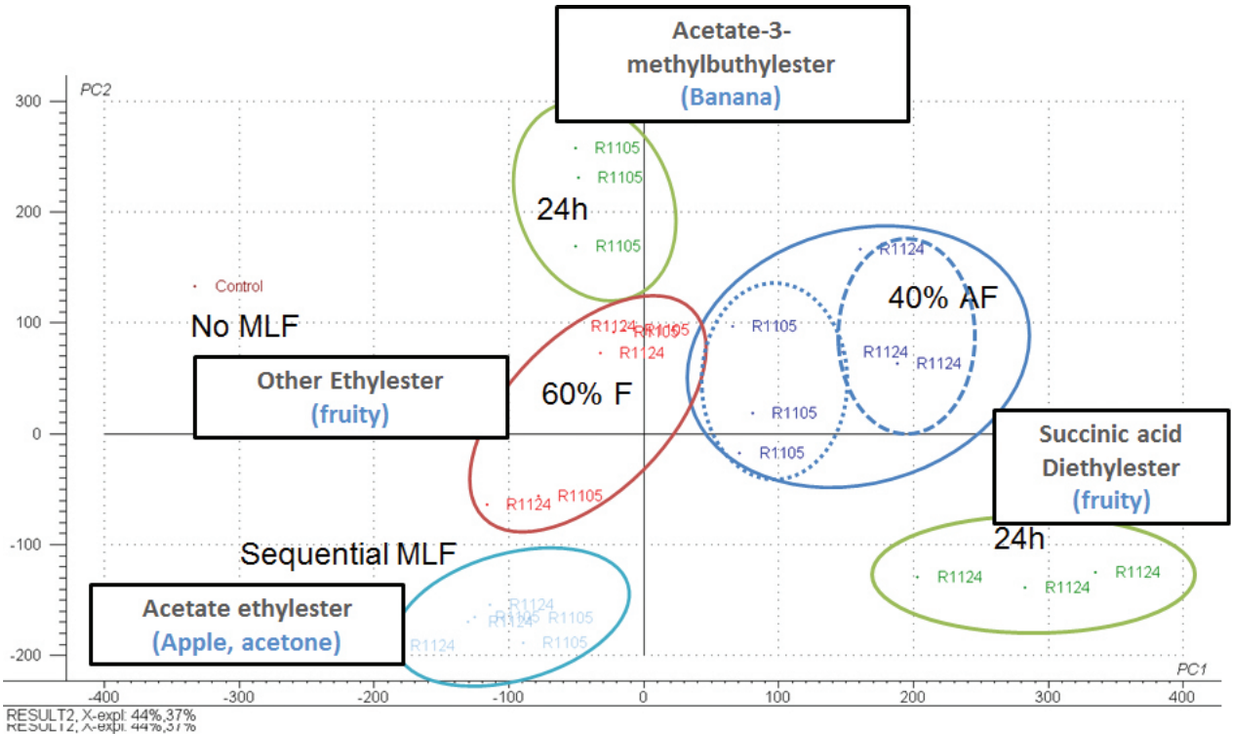

Figure 7: Principle Component Analysis score plot derived from volatile aroma compounds of all Riesling wines following MLF and the control wine with no MLF (Adapted from Knoll et al. 2012)

Impact of pH and ethanol

Knoll et al. (2011) investigated the influences of pH and ethanol on MLF and the volatile aroma profile in Riesling and Chardonnay using two different *O. oeni* strains. The results demonstrated that even if the MLF was incomplete (low pH and high ethanol), the ester concentrations were impacted. An increase in fruity esters such as ethylacetate, ethylpropionate and ethylbutyrate was observed. Acetic acid ethylester, acetic acid 3-methylbutylester, succinic acid diethylester or lactic acid ethylester were most affected by wine pH and ethanol. Lower pH resulted in greater increases in total fruity esters. For monoterpenes the content of trans- and cis-linalooloxide and α-terpineol increased with lower pH values and the linalool content increased with higher pH.

WHAT DOES THIS MEAN FOR THE WINE INDUSTRY?

Increased knowledge and a better understanding of the role of LAB in winemaking, the diversity of species and strains available and the development of different MLF inoculation scenarios have generated endless possibilities for alternative LAB to be employed as MLF starter cultures in future. The first alternative MLF starter culture using *O. oeni* or *L. plantarum* as single strains was researched by my MLF group with *L. plantarum* and *O. oeni* being combined for co-inoculation in high-pH wines. This led to commercialising Co-Inoculant by Anchor Yeast/Oenobrands and showed that you could combine the old and new (Lerm et al. 2012). The second strain released in 2014 from this group is NoVA™ by ChrHansen whereby an *L. plantarum* strain

from the Institute for Wine Biotechnology will be used to inoculate for MLF before AF aimed at no- or low-sulphur wines.

FUTURE PERSPECTIVES

The increase in wine LAB genomes being sequenced will provide information that can be used to better select strains for specific traits or to optimise the performance of other strains. We are currently sequencing the genomes of three *O. oeni* and two *L. plantarum* wine isolates. The availability of genomic, transcriptomic and metabolomic tools for LAB will allow us to study the impact of wine parameters on LAB holistically and also to assess the genetic interaction of yeast and bacteria. The research on LAB and their role in wine is far from over and in some respects has just started.

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