The effect of linoleic acid on *Saccharomyces cerevisiae*

FRANCESCA CASU

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Abstract

During fermentation *Saccharomyces cerevisiae* metabolises the nutrients present in the surrounding environment and secretes metabolic products. Therefore, its metabolism together with the substrate composition play an important role in determining the characteristics of the final fermented products. For example, during Sauvignon blanc wine making, a polyunsaturated fatty acid present in the grape juice, linoleic acid, affects the development of aroma compounds and other properties of the wine fermented by *S. cerevisiae*. However, only one single *S. cerevisiae* strain was used to ferment the wine and it is not known if linoleic acid has a similar effect on other strains. Moreover, previous research focused on the impact of linoleic acid on the wine profile rather than on the cellular pathways. For these reasons, the effect of linoleic acid on the overall cell metabolism is still not clear. This project focused on unlocking the metabolic response of *S. cerevisiae* to linoleic acid during wine making and in a laboratory-controlled environment using metabolomics and lipidomics platforms. These approaches provided an overall idea of how linoleic acid affects the wine fermented by different wine yeast strains (*S. cerevisiae* EC1118, AWRI796 and VIN13) and which cellular pathways were involved. Firstly, I investigated the effect of linoleic acid on the development of aroma compounds and other metabolites of Sauvignon blanc wines. Linoleic acid clearly affected the levels of acetylated aroma compounds, several amino acids, and antioxidant molecules, independent of the yeast strain used for fermentation. The analysis of the resulting wines provided an indirect evidence of the linoleic acid effect on *S. cerevisiae* without clarifying which yeast metabolic pathways were affected. In order to investigate this, the yeast cells were cultured on glucose supplementing them linoleic acid, and intracellular and extracellular profiles were determined using mass spectrometry. The transport of linoleic acid into the cells had an impact on primary carbon metabolism increasing glucose consumption and ethanol production, thus accelerating the fermentation rate. The energetic state of the cells was therefore affected and the glycolytic pathway, the TCA cycle and the amino acid production

were up-regulated. Moreover, since the *S. cerevisiae* fatty acid profile was altered, an experiment in parallel was performed supplementing the medium with a labelled isotope of linoleic acid to follow its metabolic fate. The finding showed that linoleic acid was metabolised into longer and shorter chain fatty acids. Since fatty acids strongly influence the cellular lipids, an analysis of the lipidome was also performed showing a general reduction of the lipid content in response to linoleic acid. Lipid biosynthesis requires ATP, therefore its reduction is due to the fact that available energy is diverted to linoleic acid uptake and compartmentalisation. The effect of linoleic acid on *S. cerevisiae* metabolism could be used by several industries. For example, during wine making, the production of aroma compounds can be manipulated through linoleic acid supplementation in the juice in order to reach a desired profile. Moreover, the biofuel industry could test the supplementation of a chosen substrate with this fatty acid, since linoleic acid improves ethanol production by *S. cerevisiae*.

To my life partner, Davide and my daughter, Sabrina

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CO-AUTHORS

Name	Nature of Contribution
Farhana R. Pinu	Assistance with experimental design, data analysis and manuscript edition
Bruno Fedrizzi	Assistance with experimental design and manuscript edition
David R. Greenwood	Assistance with experimental design and manuscript edition
Silas G. Villas-Bôas	Assistance with experimental design and manuscript edition

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Farhana R. Pinu	Farhana R Pinu	01/11/2016
Bruno Fedrizzi		04/11/2016
David R. Greenwood		2/11/2016
Silas G. Villas-Bôas		01/11/2016

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CO-AUTHORS

Name	Nature of Contribution
Farhana R. Pinu	Assistance with experimental design, data analysis and manuscript edition
Eliezer Stefanello	Assistance with experimental design and experiments
David R. Greenwood	Assistance with experimental design and manuscript edition
Silas G. Villas-Bôas	Assistance with experimental design and manuscript edition

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The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Farhana R. Pinu	Farhana R Pinu	01/11/2016
Eliezer Stefanello	Eliezer Stefanello	03/11/2016
David R. Greenwood		2/11/2016
Silas G. Villas-Bôas		01/11/2016

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CO-AUTHORS

Name	Nature of Contribution
Farhana R. Pinu	Assistance with experimental design, data analysis and manuscript edition
Sergey Tumanov	Assistance with experiments, data analysis and manuscript edition
David R. Greenwood	Assistance with experimental design and manuscript edition
Silas G. Villas-Bôas	Assistance with experimental design and manuscript edition
ELIEZER STEFANELLO	Assistance with experiments and experimental design

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- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Farhana R. Pinu	Farhana R Pinu	01/11/2016
Sergey Tumanov		9/11/2016
David R. Greenwood		2/11/2016
Silas G. Villas-Bôas		01/11/2016
ELIEZER STEFANELLO	Eliezer Stefanello	03/11/2016

CHAPTER ONE

Introduction and literature review

1.1. Winemaking: the origins

Evidences of humans intentionally fermenting grape juice to make wine, date back to as early as 7000 BC (McGovern et al., 2003; McGovern et al., 2004). This drink which would not spoil and could be stored for long time with a particular and pleasant psycotropic effect (Pretorius et al., 2012) became popular through the Roman Empire, Greece and Northern Europe first, and in the 16th and 17th century, and after the America discovery it also reached the new continent. The grape commonly used to make wine belongs to the Eurasian species *Vitis vinifera* which comprehends thousands of varieties (Alleweldt & Dettweiler, 1994; Levadoux, 1956). However, only a few of them are nowadays commercially used in the wine industry.

1.2. Sauvignon blanc in the world

Many varieties originated from France, as the Sauvignon blanc, which is the third most planted wine variety in the French regions. In particular, Sauvignon blanc is originated from the Bordeaux region in France (MacNeil, 2001) and it is a green grape. Nowadays this grape is broadly cultivated and Sauvignon blanc wine is produced not only in France but also in USA, New Zealand, Chile, South Africa, Italy, Australia and a less significant amount in a few other countries (Robinson et al., 2013). Previous research showed that the wine aroma is strongly dependent on factors such as the “terroir” (Carey et al., 2008; Sharpe, 2005), the seasonal variation (Caven-Quantrill & Buglass, 2008) and the vineyards management and practices (Trought et al., 2008). Sauvignon blanc, for instance, according to the country and the region of origin, shows different characteristics in the aroma profile (Lund et al., 2009). Furthermore, other players that determine the wine properties are the juice and the yeast. In the winemaking process, the grapes are squeezed, and the grape juice is fermented by yeasts to produce wine. In ancient times the grape juice was left to ferment with the yeasts naturally present on the grapes (Pretorius, 2000). However, this is an uncontrolled and non-reproducible way to make wine due to the many different species

that are available on the grapes (Pretorius, 2000). In fact, it is well known that each yeast species, or even strain, can affect the wine aroma and characteristics in different ways (Lambrechts & Pretorius, 2000). For this reason, nowadays the wine industry prefers to inoculate the juice using a single species of yeast. The choice of the yeast species and strain is extremely important because it will determine the aroma and flavour of the final product, the wine.

1.3. New Zealand Sauvignon blanc

In New Zealand, Sauvignon blanc became quickly popular even if it was only planted for the first time in the 1970s. At the end of the twentieth century, the Marlborough region at the north of the South Island experienced an extraordinary expansion of the vineyard despite the short Sauvignon blanc history of the country. Marlborough SB style was even claimed to be a varietal benchmark because of its particular aroma and flavour (Gregutt, 2007). In fact, the comparison of Sauvignon blanc wines produced in several countries showed that the Marlborough SB has a unique and distinctive aroma profile with tropical and passion fruit characteristics and higher concentrations of specific aroma compounds (Lund et al., 2009). The Marlborough SB quickly became very popular abroad (**Figure 1.1**) and it represents the most exported New Zealand wine in 2015 with a value of more than a billion dollars per year (Winegrowers, 2015).

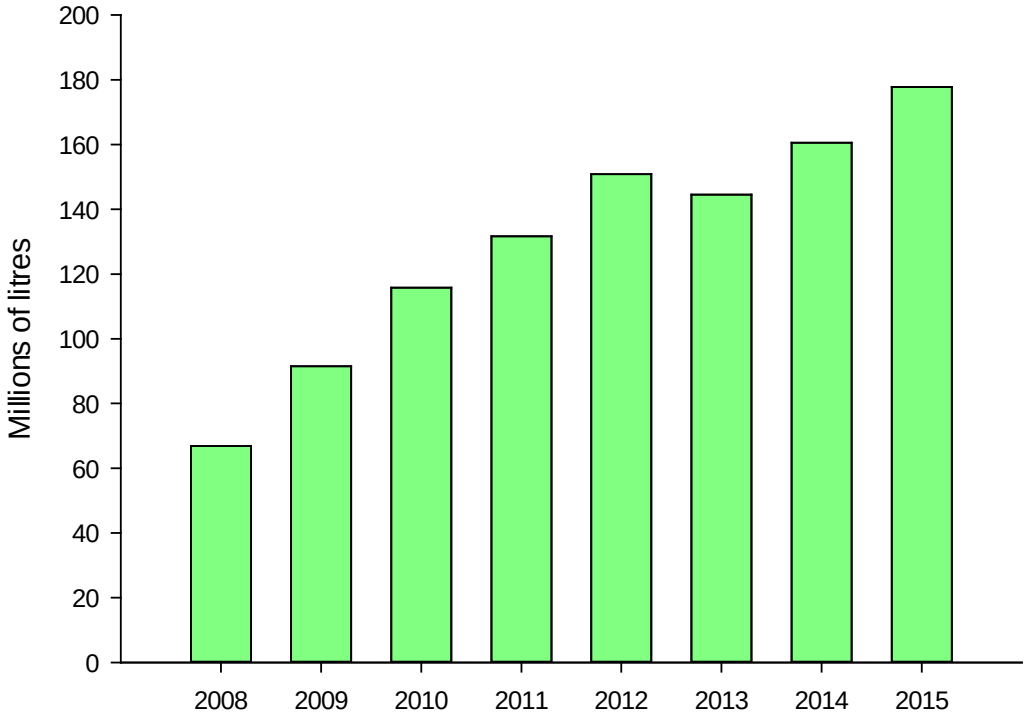


Figure 1.1. New Zealand Sauvignon blanc wine exported from 2008 to 2015 (Winegrowers, 2015).

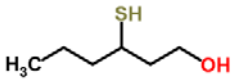
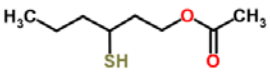
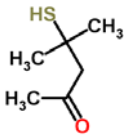
1.4. Sauvignon blanc juice and wine

The yeast fermentation transforms the grape juice in wine. The grape juice is a complex matrix very rich in sugars and nutrients. The juice composition can vary according to many elements, such as the region, the vineyard practice, the season, the weather, the ripening of the fruits and others. Among the nutrient present in the juice, there are sugars (mainly glucose and fructose), amino acids, organic acids, lipids, minerals, vitamins and other secondary metabolites (Huang & Ough, 1991; Mato et al., 2007). The yeast grows transforming the sugars into ethanol, glycerol and organic acids, and using the nutrients contained in the grape juice. After the yeast fermentation many nutrients are consumed and others are produced, such as organic and inorganic molecules, and volatile metabolites which confer to the newly produced beverage a characteristic flavour and

texture (Ebeler & Thorngate, 2009; Roland et al., 2012). The aroma compounds in the wine may originate either from the juice or from the yeast metabolism and they are organic acids, higher alcohols, carbonyl compounds, sulphur-containing compounds, phenolic compounds and volatile esters (Saerens et al., 2010).

The fruity aroma of the Sauvignon blanc depends on the concentration of three volatile thiols, esters, higher alcohols, methoxypyrazines and terpenes (Benkwitz et al., 2012). The volatile thiols, 3-mercaptohexan-1-ol (3MH), 3-mercaptohexyl acetate (3MHA) and 4-mercapto-4-methylpentan-2-one (4MMP), are respectively responsible for grapefruit, passion fruit and box tree aromas (Benkwitz et al., 2012; Nicolau et al., 2006; Tominaga et al., 1998) (**Table1.1**).

Table 1.1. Structure and olfactory description of volatile thiols in Sauvignon blanc wine (modified from Pinu (2013))

Name	Structure	Olfactory description
3-Mercaptohexanol (3MH)		Grapefruit
3-Mercaptohexyl acetate (3MHA)		Passion fruit
4-mercapto-4-methylpentan-2-one (4MMP)		Box tree, cat's pee

Marlborough SB has unique concentrations of the volatile thiols compared to SB produced in other countries of the world, which confer a particular tropical and passion fruity flavour (Lund et al., 2009). The volatile thiols are not present in the grape juice but they appear in the wine during the fermentation (Dubourdieu et al., 2006; Swiegers et al., 2009), which means that they are synthesised during this process by the yeast. Conversely,

a very small amount of 3MH was reported in grape juice (Capone et al., 2011), which could be produced by yeasts naturally present on the grapes before the actual wine fermentation. The volatile thiols synthesis during wine making has been widely studied. For instance, the volatile thiol 3MHA is the product of the acetylation of 3MH catalysed by the alcohol acetyltransferase ATF1 (Swiegers et al., 2006). However, the synthesis of 3MH and 4MMP still needs to be completely clarified. Several putative precursors have been proposed. Among these we can find cysteinylated compounds such as S-4-(4-methylpentan-2-one)-L-cysteine (Cys-4MMP) and S-3-(hexan-1-ol)-L-cysteine (Cys-3MH) (Tominaga et al., 2000; Tominaga et al., 1998), glutathionylated precursors, such as 3-S-glutathionylhexan-1-ol (GSH-3MH) (Roland et al., 2011) and S-3-(4-mercapto-4methylpentan-2-one)-glutathione (GSH-4MMP) (Fedrizzi et al., 2009), 3-S-cysteineglycine-3MH (Cys-gly-3MH) (Peyrot des Gachons et al., 2002), (*E*)-2-hexenal (Roland et al., 2011), mesityl oxide (Schneider et al., 2006). Unfortunately, the low conversion efficiency (Allen et al., 2011; Patel et al., 2010) and the lack of direct correlation of these precursors with 3MH and 4MMP (Dubourdieu et al., 2006; Roland et al., 2010; Subileau et al., 2008) leave the mystery unsolved. However, in all these studies, the research of precursors was focused on compounds, which could be somehow close to the volatile thiols, such as sulfur or sulfur related compounds. Recently, an untargeted and unbiased analysis was performed using a metabolomics platform which broaden up the research for potential precursors to other molecules (Pinu et al., 2014b). In fact, it was revealed for the first time an interesting correlation between a polyunsaturated fatty acid, the linoleic acid and the volatile thiols levels in the wine (Pinu et al., 2014b). Further studies need to be done to unravel the link between linoleic acid and the volatile thiols.

1.5. The yeast and its role in winemaking

Many yeasts are able to ferment (Van Dijken et al., 1986) but just a few of them can grow under anaerobic conditions including *Saccharomyces cerevisiae* (Visser et al., 1990). Thanks to this property *S. cerevisiae* is extensively used, in fact, it is the almost

exclusive species used in wine making (Pretorius, 2000). The grape must, although rich in nutrients, has a very high sugar content and a low pH, therefore it is a very selective medium for organisms to grow. Furthermore, the winery practices, such as the addition of sulphur dioxide as antioxidant and antimicrobial (Henschke, 1997), and the lack of oxygen, make the grape must a very challenging medium to grow in. In this environment *S. cerevisiae* is able to quickly and efficiently convert the sugars into ethanol, carbon dioxide and many other metabolites which contribute to the final wine aroma and flavour (Pretorius, 2000). *S. cerevisiae* species comprehends many strains and according to the strain used to ferment the grape juice, the wine will have different characteristics (Lambrechts & Pretorius, 2000). An example is Sauvignon blanc wine, which shows different aroma profiles depending on the fermenting strain (Dubourdieu et al., 2006, **Table 1.2**). The responsible of this effects on the wine is the metabolism of *S. cerevisiae,* which changes according to the strain and also according to the nutrients provided. In fact, the metabolites production can be affected by physical or chemical conditions, such as a temperature change or an alteration of the nutrients, consequently, up- or down-regulating the cellular pathways.

Table 1.2.. Yeast strain effect on 4MMP, 4MMPOH, and 3MH in four Sauvignon blanc wines after alcoholic fermentation (1999 vintage). Modified from Dubourdieu et al. (2006).

	Wine[a]				
	1	**2**	**3**	**4**	**Average**
4MMP (ng/L)					
VL3c	12	12	12	10	12 a
EG8	8	9	16	8	10 a
VL1	7	2	7	6	6 b
522d	0	0	0	0	0 c
4MMPOH (ng/L)					
VL3c	28	12	27	41	27 a
EG8	25	9	10.6	39	21 ab
VL1	25	7	9	38	20 ab
522d	25	6	2	32	16 b
3MH (ng/L)					
VL3c	2161	3261	413	991	1706
EG8	2894	4581	460	1135	2267
VL1	2077	2227	305	1457	1516
522d	2128	2890	235	1184	1609

Values followed by different letters are statistically different ($p < 0.01$, ANOVA)

1.6. Fatty acids and linoleic acid isomers

Fatty acids are carboxylic acids with an aliphatic chain and they are mostly unbranched. They can be saturated if they do not have double bonds, monounsaturated if they have one, polyunsaturated (PUFA) if they have more than one double bond (**Figure 1.2**). Their carbon atoms can be counted from the carboxylic group or from the methyl-end group. In the first case, the carbon atoms are called C1, C2, C3… etc., in the second case their position is specified by ωn. The double bonds, therefore, can be defined by Δ^n or ωn according to which side of the molecule they are counted. In literature, most fatty acids are referred to by their common names rather than the IUPAC name and the lipid numbers are also commonly used. The lipid numbers have the form *C:D* where *C* refers to the number

of carbons and *D* is the numbers of double bonds. In the case of isomers this notation needs the Δ^n or ωn specification to eliminate ambiguities. Short chain fatty acids (SCFA) have up to 5 atoms of carbon, medium chain fatty acids (MCFA) have from 6 to 12, long chain fatty acids have from 13 to 22 (LCFA) and very long chain fatty acids (VLCFA) have over 23 atoms of carbons.

The biological functions of the fatty acids are very diverse. They are structural components of the cellular membranes but they are an energy reserve as well because their catabolism produces ATP. Some of them also act as cofactors, electron carriers, hydrophobic anchors for proteins, hormones and intracellular messengers (Lehninger et al., 2005). There is an emerging interest around their role in eukaryotic cells which is a result of the increasing number of studies on lipid analysis and lipidomics (Wenk, 2005). For example, in the wine making field, interesting observations have been done regarding the amount of lipids in the grape juice. It was observed that, although it is a water based matrix, Sauvignon blanc juice has a total lipid content as high as 2.80 g/L (Tumanov et al., 2015), which is an important amount of nutrients for the fermenting yeast. The fatty acids identified in this study were 15 including eight polyunsaturated fatty acids. The latter are particularly relevant in juice fermentations, because *S. cerevisiae* is not able to synthesise them under anaerobic conditions and they have to be taken up from the environment to be used for the yeast growth (Moonjai et al., 2003). *S. cerevisiae* growth though, is not the only affected factor, a correlation between linoleic acid present in the Sauvignon blanc juice and the concentration of some wine aroma compounds was observed (Pinu et al., 2014b).

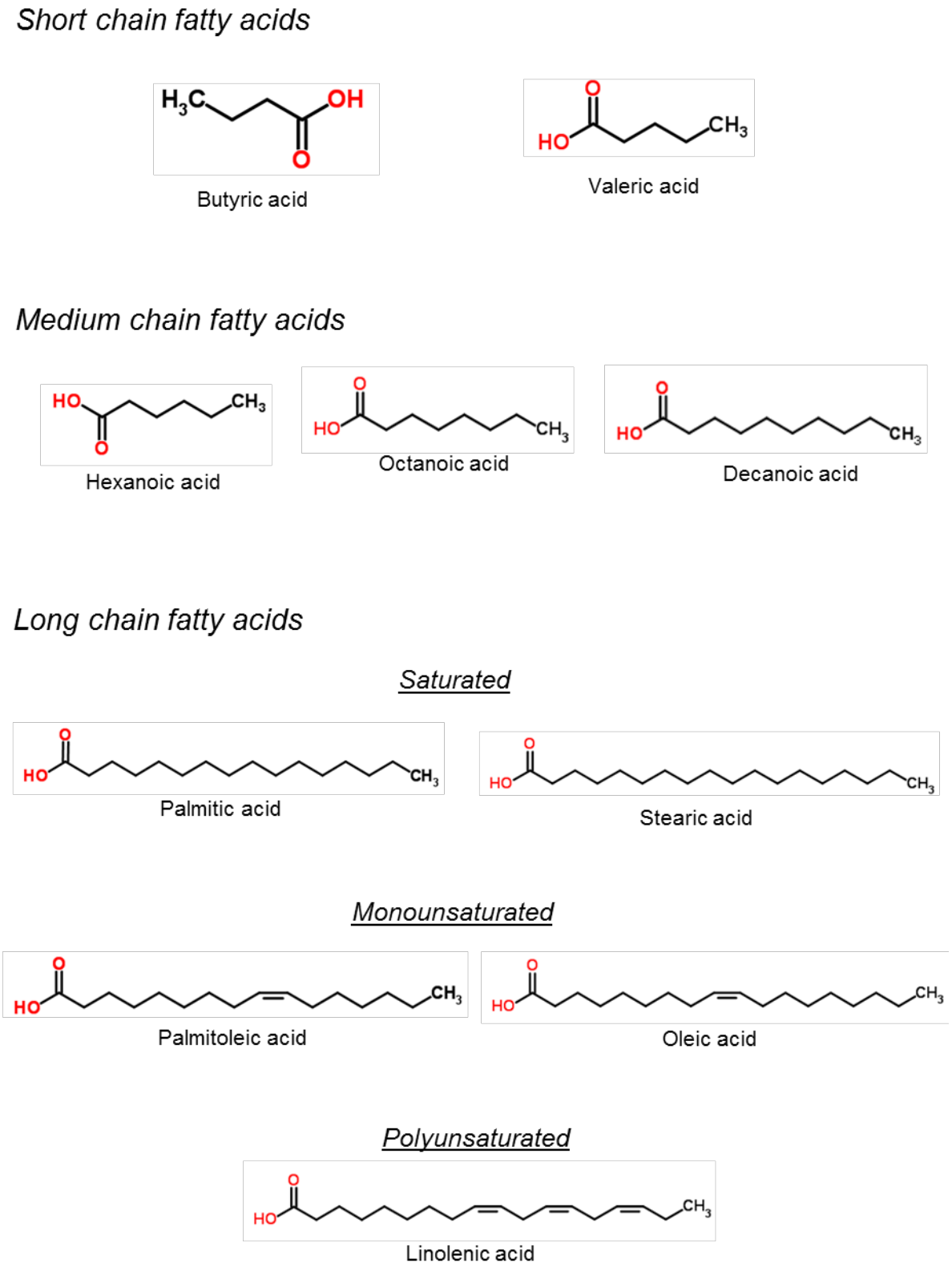


Figure 1.2. Examples of fatty acids.

1.6.1. Linoleic acid and its influence on the wine properties

The linoleic acid is a polyunsaturated fatty acid made of 18 atoms of carbon with two *cis* double bonds on the 9th and the 12th position. *De novo* PUFAs biosynthesis cannot be performed by *S. cerevisiae* in any condition, in fact this yeast can only synthesise monounsaturated fatty acids, mainly palmitoleate (16:1) and oleate (18:1), and exclusively in presence of oxygen (Daum & Vance, 1997; Martin et al., 2002; Stukey et al., 1989, **Figure 1.3**).

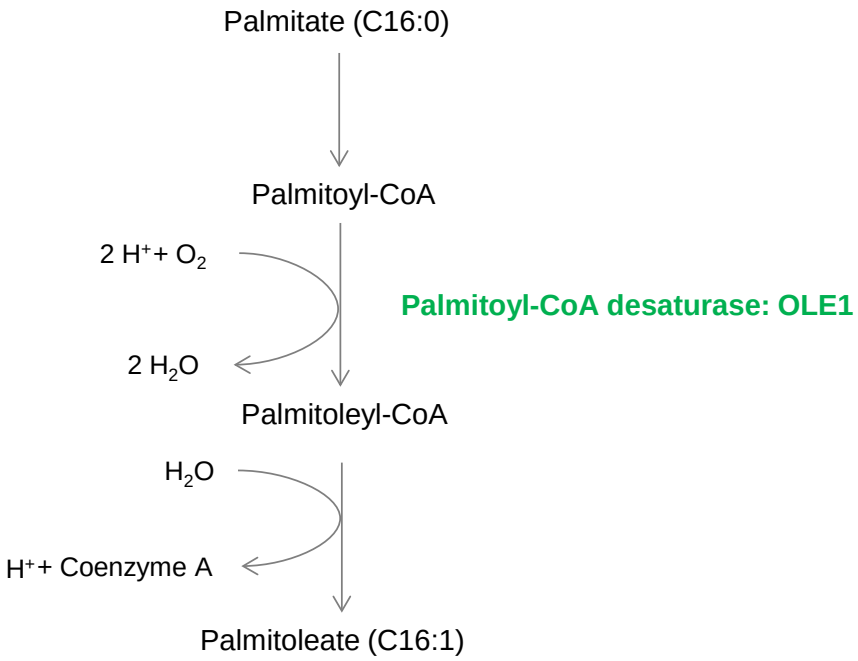


Figure 1.3. *Saccharomyces cerevisiae* biosynthesis of a monounsaturated fatty acid. The example here is the palmitoleate. The double bond is created by a desaturase in an oxygen dependant manner. The other monounsaturated fatty acids are synthesised in the same way (Stukey et al., 1989).

PUFAs degradation instead, can be achieved by *S. cerevisiae* under aerobic conditions breaking them down into the peroxisome via β-oxidation (Hiltunen et al., 2003, **Figure 1.4**). However, the fatty acids β-oxidation does not occur under anaerobic conditions, as in wine making, due to the lack of oxygen. For these reasons, during wine fermentation, *S. cerevisiae* cannot synthesise PUFAs and cannot break down fatty acids therefore its growth strongly depends from their uptake from the environment.

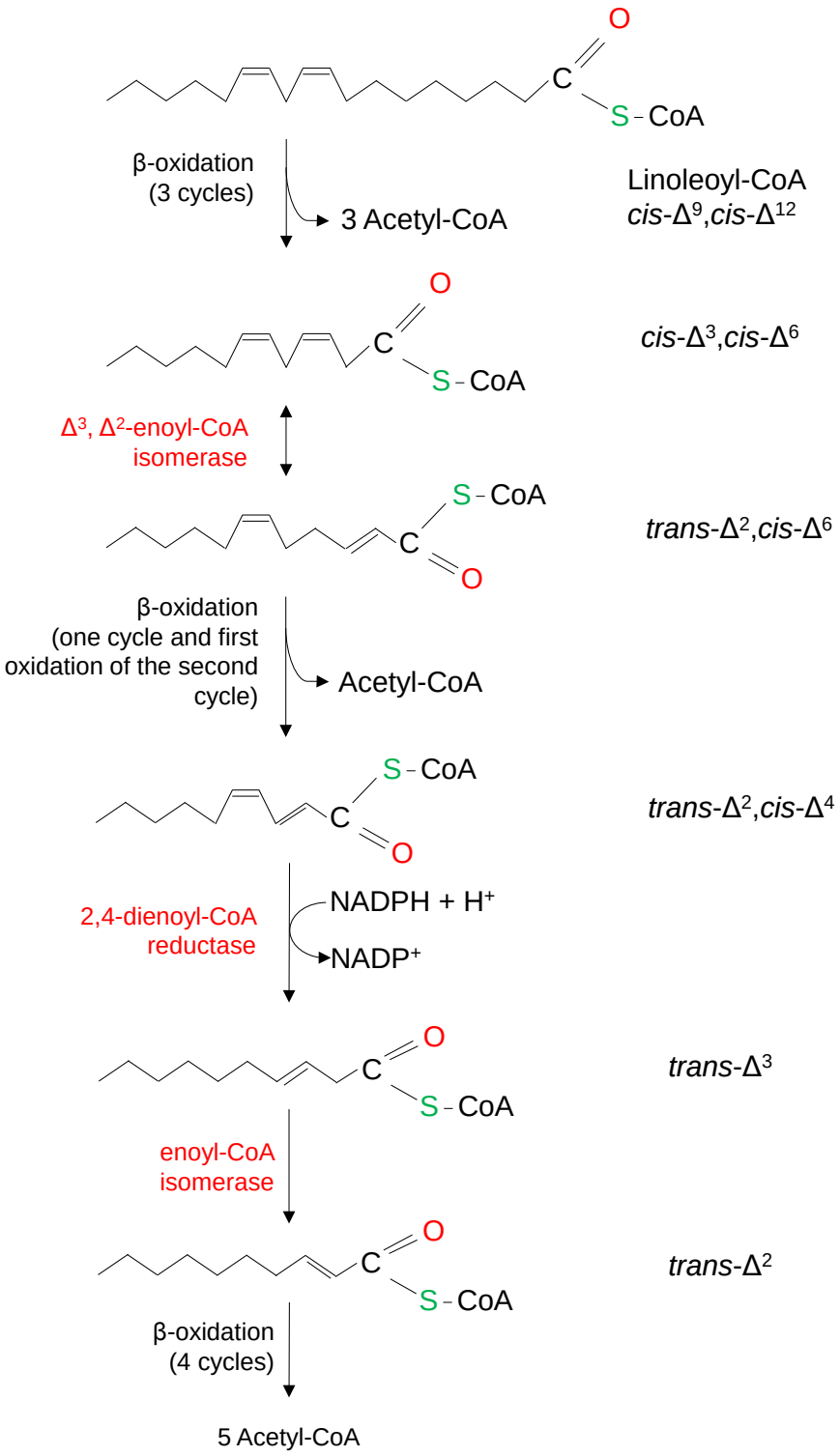


Figure 1.4. Oxidation of a polyunsaturated fatty acid. The example shows the linoleic acid. In addition to the oxidative steps, the action of an isomerase and a reductase is required to complete the polyunsaturated fatty acid oxidation. Modified from Lehninger et al. (2005).

Linoleic acid in the grape juice affects *S. cerevisiae* aroma compounds production (Dubourdieu et al., 2006; Swiegers et al., 2009), therefore influencing the yeast metabolism. For example, it was observed that *S. cerevisiae* produces less acetylated esters when linoleic acid was supplemented to the media in anaerobic conditions (Fujiwara et al., 1999; Malcorps & Dufour, 1992). The reason for the *S. cerevisiae* reduced production of these important aroma compounds is that linoleic acid inhibits a gene which encodes an alcohol acetyltransferase, ATF1 (Fujiwara et al., 1999; Moonjai et al., 2002). This acetyltransferase is responsible for the acetylation of many compounds, acetate esters included. Therefore, *S. cerevisiae*, in presence of linoleic acid, produces less acetate esters. Previous studies showed that, the supplementation of linoleic acid to the must decreased *S. cerevisiae* production of a Sauvignon blanc volatile thiol, 3MHA (Pinu et al., 2014b). 3MHA is the product of the 3MH acetylation by an alcohol acetyltransferase (Swiegers et al., 2006), which is inhibited in presence of linoleic acid (Fujiwara et al., 1999; Moonjai et al., 2003; Pinu et al., 2014b). Therefore, the cells produced less 3MHA. However, even though the link between linoleic acid in the Sauvignon blanc grape juice and the aroma compounds concentrations was demonstrated, many other questions arise. For instance, only one *Saccharomyces cerevisiae* strain was tested, and it would be interesting to verify if linoleic acid had the same effect on other commercially used wine yeast strains as well. We are also not aware if different linoleic acid concentration would have the same or a similar effect on *S. cerevisiae* metabolism.

1.6.2. Conjugated linoleic acid

In nature, molecules with the same chemical formula and the same mass, but with different atom arrangement may have very different roles. These are known as isomers. The linoleic acid and its isomers, the conjugated linoleic acids (CLAs), are an example of extremely similar compounds, which carry different properties. The conjugated linoleic acids group comprehends 28 isomers (Banni, 2002). CLAs has been investigated broadly in the last years for their beneficial properties on the health. Previous research observed

that CLAs have effects against carcinogenesis, obesity, diabetes, inflammation, atherosclerosis and they also help to promote the bone formation (Belury, 2002; Bergamo et al., 2014; Druart et al., 2014; Hwang et al., 2006; Jaudszus et al., 2005; Kennedy et al., 2010; Kim et al., 2014; Lee et al., 1994; McGowan et al., 2013; Park et al., 1997; Racine et al., 2010; Tricon et al., 2004; Valeille et al., 2006). The majority of the studies were performed using mixture of CLA isomers, usually mainly c9,t11-CLA and t10,c12-CLA (Kelley et al., 2007) with minor concentration of other isomers. These two isomers c9,t11-CLA and t10,c12-CLA are the only ones known to possess biological activity (Pariza et al., 2000). In particular, c9,t11-CLA is the main isomer found in nature (Pariza et al., 2001) and it is also called rumenic acid because it can be biosynthesised from linoleic acid by ruminant microorganisms, i.e. *Butyrivibrio fibrisolvens* (Kepler et al., 1966). c9,t11-CLA can also be synthesised using vaccenic acid as substrate in the mammary gland (Bauman et al., 2001) and it is accumulated in the milk fat and in the ruminants' tissues. Moreover, other food-grade bacteria are able to synthesise c9,t11-CLA, such as some species belonging to the genus *Bifidobacterium* (Barrett et al., 2007; Gorissen et al., 2010; Rosberg-Cody et al., 2011), *Lactobacillus* (Hosseini et al., 2015; Yang et al., 2014), *Lactococcus* (Ogawa et al., 2005) and *Propionibacterium* (Hennessy et al., 2012). The commercial interest around the production of CLA led to an effort to engineer *S. cerevisiae* with the aim to produce CLA. *S. cerevisiae* is able to synthesise *de novo* saturated and mono saturated fatty acids (Daum & Vance, 1997; Martin et al., 2002) but not PUFAs. The insertion of exogenous genes is necessary for the yeast to produce PUFAs (Dyal & Narine, 2005; Veen & Lang, 2004). Although the biological effect of CLA was studied concerning its effect on human diseases, not many information are available regarding its effect on *S. cerevisiae* except that the yeast is able to grow on plate using *trans*-10,*cis*-12 CLA but not *cis*-9,*trans*-11 CLA[65]. Evidences indicate that its isomer, LA, has an effect on the yeast metabolism but whether CLA has an effect it is still unclear.

1.7. Metabolomics

Metabolomics refers to a series of analytical platform that uses an untargeted and unbiased approach to analyse metabolites in biological samples (Villas-Boas, 2013; Villas-Bôas et al., 2005). It was first proposed as analytical strategy in functional genomics (Oliver et al., 1998) and it is part of the "omics" technologies. The genomics, transcriptomics and proteomics are focused on the DNA, the RNA and the proteins, respectively, while the metabolomics studies the metabolites. The totality of the metabolites in a specific sample is defined as metabolome and being the downstream product of the cell metabolism it can be considered the phenotype of the cell (Fiehn, 2002). However, the metabolome is very complex and chemically diverse. Therefore at the moment we are not able to analyse the whole set of cell metabolites (Dunn et al., 2013; Villas-Bôas et al., 2005). The metabolites are small molecules (<1500 Daltons) belonging to a broad range of classes. In contrast to the target metabolite analysis, which employs specific methods for a single molecule or a class of compounds, metabolomics uses several analytical platforms to cover the analysis of as many metabolites as possible. Moreover, the metabolomics data analysis requires powerful bioinformatics tools to process spectrums and chromatograms for all detected analytes (Gummer et al., 2009). Recently, thanks to the progress in separation and identification of metabolites (Wishart, 2008), a metabolite profiling can be obtained by coupling separation methods (gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE)) with detection methods (mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR)) (Koek et al., 2011). A metabolite profile includes both identified and unknown molecules (Shulaev, 2006), which is an advantage in comparison to the target analysis, for the discovery of new compounds or in their association to the biological question. However, some challenges and limitations remain; in fact not all detected metabolites are identified leaving many compounds unknown. Moreover, differently from the targeted approaches that obtain the absolute quantification using calibration-curves of standards, the metabolomics techniques are mainly semi-quantitative. However, recently, new methods for quantification of amino and non-amino

organic acids were developed (Kvitvang et al., 2011; Tumanov et al., 2016), and these novel approaches can be a starting point for the quantification of hundreds of metabolites.

1.7.1. GC-MS based metabolomics

The metabolome comprehends thousands of metabolites very diverse in matter of polarity and molecular weight (Shulaev, 2006) and the GC-MS is one of the analytical platforms most commonly adopted to study it (Villas-Boas, 2013). The necessity of a high-throughput metabolite profiling coupled the efficient GC separation with the high sensitivity of the MS detector (Glinski & Weckwerth, 2006).

Mass spectrometers detectors measure the mass of electrically charged compounds as mass to charge ratio (m/z) and give information regarding the structure, purity and composition of metabolites. A mass spectrometer has four main components: the sample introduction system, the ion source, the mass analyser and the detection system. Powerful computers are required in order to control the electronic and physical part of the MS and to process the output data.

GC-MS (**Figure 1.5**) involves the sample to be introduced using a chromatographic separation in a 10 to 60 m column with an internal diameter ranging from 100 to 500 µm. This type of columns require a very small amount of sample (1-2 µL) and the mobile phase is an inert gas, such as hydrogen, helium, nitrogen. The separation takes place at high temperatures and the analytes are volatiles in the gas phase. The ionisation source charges each metabolite by removing an electron. In this form the ions can be analysed by the mass analyser. Several types of ion sources can be used: electrospray ionisation (ESI), electron impact ionisation (EI), chemical impact ionisation (CI), etc. In metabolomics, the mainly used ionisations are EI and ESI. EI only operates under vacuum pressure. Even though it is largely used it cannot ionise large molecules (Kitson et al., 1996). On the other hand, ESI operates at atmospheric pressure and thanks to the nanospray technology which operates at low flow rate a wide range of metabolites can be analysed (Karas et al., 2000).

When the ions reach the mass analyser they are separated according to their m/z values. On the market there are available several kinds of mass analysers: quadrupole (Q), quadrupole ion-trap (QIT), time of flight (ToF), orbitrap, ion mobility spectrometry (IMS) and Fourier transform ion cyclotron resonance (FT-ICR). The quadrupole mass analyser are considered robust, low cost and easy to use even although the mass resolution and accuracy compared to the other mass analysers is lower (Villas-Bôas et al., 2005).

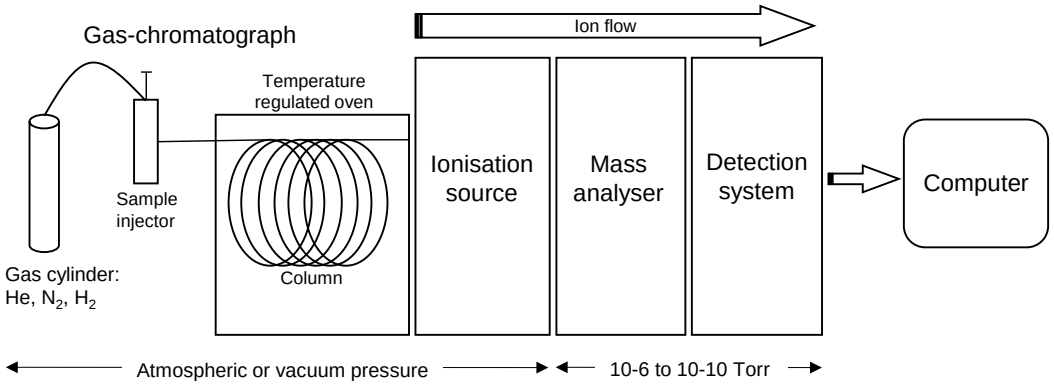


Figure 1.5. Schematic configuration of a GC-MS system.

A GC-MS system can be used to analyse volatile compounds, molecules with a boiling point lower than 300°C, or non-volatile compounds. In the first case the sample can be directly injected in the GC, whereas the non-volatile compound need to be acquire the volatility through a process called chemical derivatisation. Two derivatisation methods are mainly used in metabolomics: silylation and alkylation (Dunn & Ellis, 2005; Villas-Boas, 2013).

Silylation is the most used derivatisation method in metabolomics. A silyl group [-$Si(CH_3)_3$] is introduced to a metabolite replacing the active hydrogen (e.g. –OH, -SH, -NH_4^+, -COOH, etc.), making the metabolite more volatile (**Figure 1.6**). Using this derivatisation method sugars, sugar alcohols, amino sugars and their derivatives can become volatile and be analysed by GC-MS. However, even though it is an easy method because requires

only one reaction step and it is quite safe (Söderholm et al., 2010), the reagents are very sensitive to moisture and some primary metabolites such as amino acids and some organic acids form unstable silylated derivatives (Kanani & Klapa, 2007; Koek et al., 2006; Noctor et al., 2007; Villas-Bôas et al., 2011).

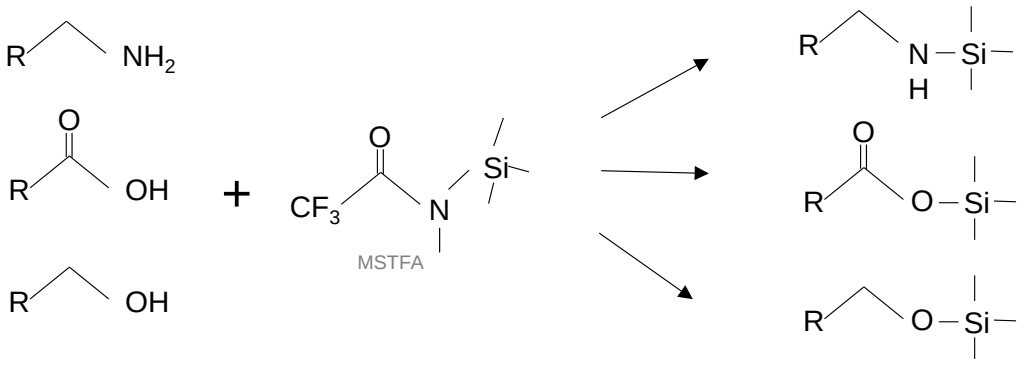


Figure 1.6. Reaction of the trimethyl silyl (TMS) derivatisation. Modified from Villas-Bôas et al. (2011)

On the other hand, the derivatisation through alkylation replaces active hydrogens of compounds with an alkyl group (**Figure 1.7**). The metabolites that can be derivatised by this method are mainly primary and secondary amines, , thiols, phenols, carboxylic acids and alcohols (Söderholm et al., 2010). The reagents used are chloroformate (CF) derivatives and among them methylcloroformate (MCF) is broadly used in metabolomics (Aggio et al., 2012; Carneiro et al., 2011; Dhami et al., 2011; Jäpelt et al., 2015; Kvitvang et al., 2011; Mudiam et al., 2013; Smart et al., 2010; Villas-Bôas et al., 2003; Villas-Boas et al., 2005; Zhao et al., 2014).

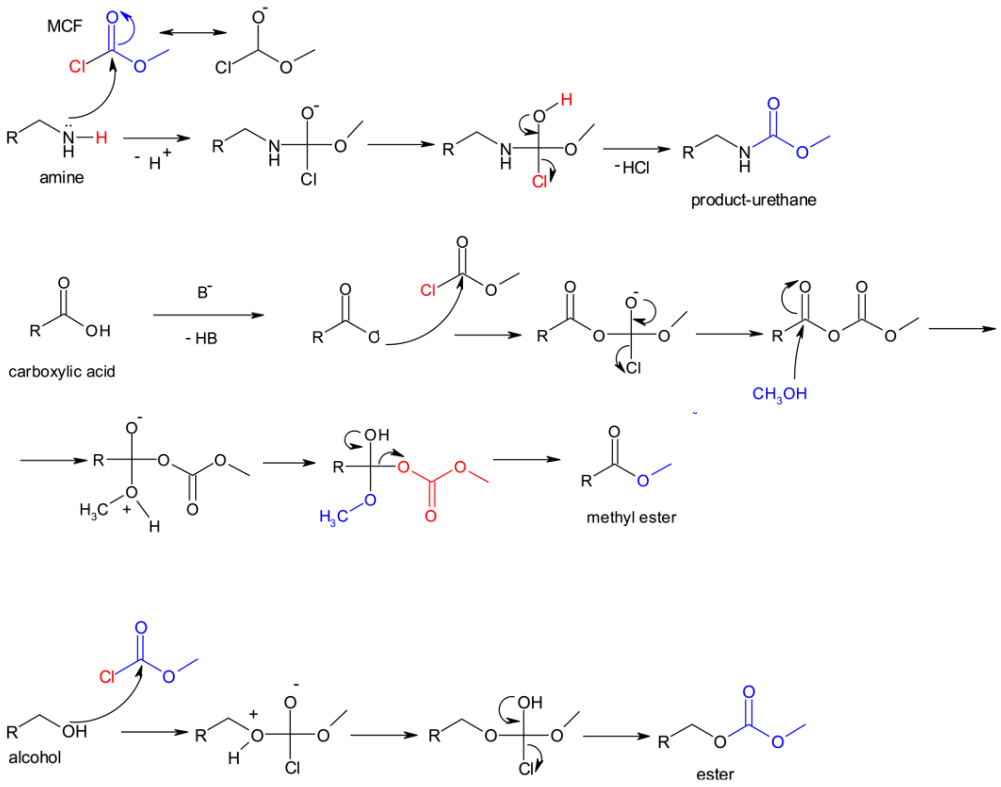


Figure 1.7. Reaction of the MCF derivatisation of three metabolites classes. Modified from Villas-Bôas et al. (2011).

The use of GC-MS systems in metabolomics has the advantage to be able to identify the metabolites using MS libraries (Koek et al., 2011; Villas-Bôas et al., 2005). The software "Automated mass spectral deconvolution and identification system" (AMDIS) is a useful tool in the identification of compounds because of its ability to deconvolute the overlapping chromatographic peaks. Furthermore, developing an in-house MS library is useful for the identification, although commercial libraries are also available to assist with the identification (Villas-Bôas & Bruheim, 2007).

1.7.2. Lipidomics

Lipidomics is a subclass of the metabolomics which studies the lipidome in a sample through qualitative and quantitative methods (Herzog et al., 2011). The lipidome comprehends all the lipid species in a sample and has such distinctive physical-chemical characteristics from polar metabolites that the development of specific analytical methods became necessary. Moreover, the lipidome covers important physiological roles (e.g. cellular membrane structures, energy storage and signalling function) (Ejsing et al., 2009) and for this reason, the scientific interest is growing (Herzog et al., 2011). Due to the complexity of the lipidome, a comprehensive classification system, called LIPID MAPS , was proposed and it is now broadly used (Fahy et al., 2009, **Figure 1.8**) .

The development in MS has been the key in the lipidomics field improvement. The mostly used ionisation sources are electrospray ionisation (ESI), electron impact (EI) ionisation and matrix assisted laser desorption/ionisation (MALDI). However, coupling an LC to an ESI mass spectrometer is so easy to make ESI largely the most applied ionisation method in lipidomics (Roberts et al., 2008). A very efficient analysis of the lipidome using ESI-MS is achieved through the direct infusion of the raw sample. For example, this platform allowed to identify and relatively quantify 450 phospholipids in mammalian cells (Milne et al., 2006). The lipid classes can be measured in positive and negative mode and the MS gives a significant separation and high sensitivity (Schiller & Arnold, 2000). The global lipidomics, also called shotgun lipidomics, aims to analyse the majority of the lipids in a sample through the direct infusion of a crude extract into the MS (Griffiths et al., 2011).

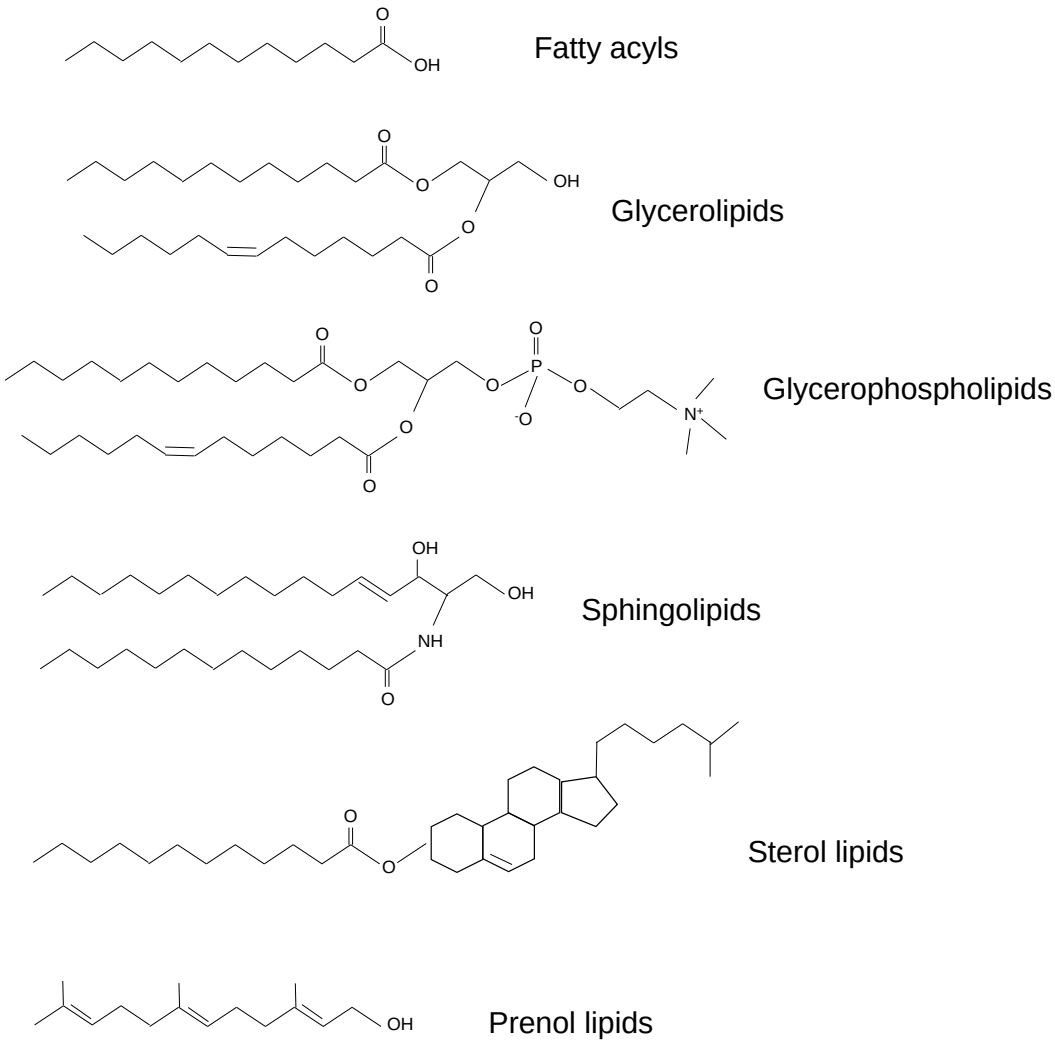


Figure 1.8. Lipid classification according to LIPID MAPS (Fahy et al., 2009).

1.7.3. The potential of metabolomics and lipidomics in wine and yeast research

The metabolites analysis in the wine has been performed mainly in a targeted way since long time (Webb, 1964). Researchers aimed to achieve the characterisation of the wine varieties studying the aroma profile using GC-MS analysis (Cobb & Bursey, 1978; Guth, 1997; Nelson et al., 1978). Similarly, the grape juice composition was analysed using

enzymatic methods or HPLC, in particular to determine the amount of sugars, amino acids and some carboxylic acids (Lafon-Lafourcade, 1977). However, this classical chemical analyses were mainly targeted approaches, aiming to study specific group of compounds that were most abundant in the samples. The modern metabolomics powerful platforms available nowadays could overcome the old obstacles allowing not only the analysis of low abundant molecules but also more comprehensive metabolic profiles (Pinu, 2013; Schmidtke et al., 2013). Moreover, metabolomics and lipidomic profiling of *S. cerevisiae,* species broadly used in wine fermentation, have been performed in different studies (Ding et al., 2009; Ding et al., 2010; Ejsing et al., 2009; Mashego et al., 2005; Wisselink et al., 2010). Despite evidences that a grape juice fatty acid, linoleic acid, influences the metabolism of one *S. cerevisiae* wine strain and therefore, some wine properties, it is still not clear if other strains would be affected in the same way. Moreover, the absence of studies investigating *S. cerevisiae* metabolic response to linoleic acid created the opportunity for an extensive research through metabolomics and lipidomics platforms, which are reported in this PhD work.

1.8. Aims and objectives

The main aim of this thesis was to investigate the effect of linoleic acid on *S. cerevisiae* cells and consequently to the wine produced by this yeast. The approach consisted in a series of fermentations in which *S. cerevisiae* was cultivated supplementing the media with different linoleic acid concentrations. At first, I studied the linoleic acid effect on different wine strains through the wine metabolomics. Afterwards, I investigated which *S. cerevisiae* pathways were affected by linoleic acid through yeast metabolomics and lipidomics.

To achieve these goals, this PhD project had the following specific objectives:

- Chapter 2. Studying the response of three *S. cerevisiae* wine strains to different concentrations of linoleic acid supplemented to the grape juice ;
- Chapter 3. Studying the metabolic response of *S. cerevisiae* to the linoleic acid supplementation in a minimal mineral medium and in controlled laboratory conditions using intracellular and extracellular metabolomics and lipidomics platforms;
- Chapter 4. Investigating the linoleic acid fate in the *S. cerevisiae* cells through the supplementation of a labelled isotope of linoleic acid.

CHAPTER TWO

The effect of linoleic acid on the Sauvignon blanc fermentation by different wine yeast strains

Francesca Casu[1], Farhana R. Pinu[2], Bruno Fedrizzi[3], David R. Greenwood[1,2] and Silas G. Villas-Bôas[1]

[1]School of Biological Sciences, the University of Auckland, 3A Symonds Street, Auckland 1010, New Zealand,

[2]The New Zealand Institute for Plant and Food Research Limited, 120 Mt Albert Rd, Sandringham, Auckland 1025, New Zealand

[3]School of Chemical Sciences, the University of Auckland, 23 Symonds Street,

Auckland 1010, New Zealand

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2.1. Introduction

New Zealand (NZ) has an excellent reputation for the production of premium quality wines with Sauvignon blanc (SB), the major wine variety having an export value over 1 billion dollars in 2014 (www.nzwine.com). The worldwide popularity of NZ SB wines is mainly due to its distinctive fruity and tropical aromas. Varietal thiols, including 3-mercaptohexanol (3MH) and 3-mercaptohexyl acetate (3MHA), are the major aroma compounds found in SB wines responsible for the passion fruit and grapefruit aromas, respectively (Lund et al., 2009). It is well known that NZ SB wines contain higher levels of these two varietal thiols compared to other SB wines produced in other parts of the world (Benkwitz et al., 2012; Lund et al., 2009). It is generally believed that varietal thiols are practically absent in the grape juice and usually develop during the fermentation process by the activity of wine yeasts (e.g. *Saccharomyces cerevisiae*) on different juice components including S-cysteinylated and S-glutathionylated precursors (Roland et al., 2011). However, the biogenesis of these varietal thiols is still under debate and very poor correlations have been found between them and their putative precursors present in juice (Pinu et al., 2012).

Grape juice is a very rich and complex medium that contains at least 200 g/L of sugars in addition to hundreds of other grape metabolites, including amino and non-amino organic acids and fatty acids with concentrations varying from ng/L to g/L (Huang & Ough, 1991; Mato et al., 2007). Although many studies have been carried out to determine the role of major compounds present in grape juice during wine fermentation (Son et al., 2009; Stines et al., 2000), only very few studies have looked into the grape metabolites found at trace levels in the juice (Pinu et al., 2014a; Tumanov et al., 2015). For instance, not much attention has been given to the lipids present in grape juice. The role of these trace molecules cannot be overlooked as they play a major role on yeast metabolism, and their initial concentration in the grape juice impacts on the production of fermentation end products. Pinu et al. (2014a) demonstrated that the increase in the concentration of a

polyunsaturated fatty acid, linoleic acid, in the SB grape juice influenced the development of different groups of aroma compounds in NZ SB wines fermented by *S. cerevisiae* EC1118. Similarly, Yunoki et al. (2005) showed that the high linoleic acid levels in red grape juice decreased the production of an important group of wine aroma compounds, the fatty acid ethyl esters by wine yeasts. Later, Tumanov et al. (2015) reported the first lipidomic study of SB grape juice, showing that its lipid content is very variable and can be as high as 2.80 g/L. They found a broad range of different lipid species with linoleic acid as the second most abundant fatty acid in the analysed grape juices. Therefore, these free fatty acids are readily available for *S. cerevisiae* cells during fermentation, potentially changing their metabolic activity and ultimately governing the characteristics of the final wine. Moreover, the incorporation of linoleic acid present in the grape juice seems to increase the viability of the yeast cells (Beltran et al., 2008). As wine fermentation is an anaerobic process and *S. cerevisiae* cannot synthesise unsaturated fatty acids under such conditions, these compounds including linoleic acid, which are naturally present in grape juice, are essential for yeast growth and metabolism during winemaking, and are consequently very important.

The main aim of this study was to investigate the effect of linoleic acid in the grape juice on SB wines, fermented by different *S. cerevisiae* strains in order to determine whether different wine yeast strains respond different to this fatty acid during wine fermentation when compared to *S. cerevisiae* EC1118.

2.2. Material and methods

2.2.1. Chemicals

All chemical reagents used in this research were analytical grade. Methanol, pyridine, anhydrous sodium sulfate, ethyl acetate, 2,3,3,3-d_4-alanine, methyl chloroformate (MCF), dimethyl dicarbonate (DMDC), ethyl propiolate (ETP), butylated hydroxyanisole (BHA), D-ribitol, and *N*-methyl-*N*-(trimethylsilyl)-trifluoroamide (MSTFA, derivatization grade) were purchased from Sigma-Aldrich (St. Louis, MO). Methanol-d_4 was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury,USA). Chloroform, and absolute ethanol (≥99.5%) were obtained from Merck (Darmstadt, Germany). Methoxyamine hydrochloride was obtained from Fluka (Steinheim, Switzerland). Thiol standards 3MH and 3MHA were procured from Interchim 75 (Montluc¸on, France) and Oxford Chemicals (Hartlepool, UK), respectively. Δ9Z,12Z-Linoleic acid was purchased from Nu Chek Prep. Inc (Elysian, USA). All solutions were prepared using Grade 1 water 77 (BARNSTEAD® NANOpure DIamondTM Water Purification System, Waltham, MA). The internal standards d_2-3-mercaptohexan-1-ol (d_2-3MH) and d_2-3-mercaptohexyl acetate (d_2-3MHA) were synthesised at the University of Auckland (Hebditch et al., 2007). 3-Mercaptohexanol-d_8 was synthesised at the University of Auckland, following the protocol reported by Pardon et al. (2008). For the analysis of 58 aroma compounds, a mixture of 10 deuterated internal standards was used. Among them, ethyl butanoate-d_5(100%), ethyl hexanoate-d_5 (100%) and ethyl octanoate-d_5 (99.9%) were synthesised at Lincoln University, New Zealand (Herbst-Johnstone et al., 2013b), while 3-methylbutyl acetate-d_3 (98.6%), *n*-hexyl acetate-d_3 (99.2%), 2-phenylethyl acetate-d_3 (99.4%), (±)-linalool-d_3 (98.8%), 3-methyl-1-butyl-1,1-d_2-alcohol (99.2%), *n*-hexyl-2,2,3,3,4,4,5,5,6,6,6-d_{11}-alcohol (99.1%) and 2-phenyl-d_5-alcohol (99.5%) were obtained from CDN ISOTOPES, Canada. Another mixture of standards that included 4-decanol (98%, Lancaster, Pelham, NH, USA), DL-3-octanol (99%, Acros Organics, Geel, Belgium), and 3,4-dimethylphenol (99%, Aldrich,Milwaukee,

WI, USA) was also used for the analysis of aromacompounds. Sodium chloride was purchased from Univar (Ajax Finechem, Taren Point, NSW, Australia).

2.2.2. Collection and storage of SB grape juices

Two SB juices from 2011 to 2014 harvest were collected from two different vineyards of Pernod Ricard situated in the Marlborough region, New Zealand. After pressing the grapes, adding SO2 (50 ppm) and cold settling, the juice was collected in 2-L bottles (Pinu et al., 2012). Juices were immediately frozen and transported to the laboratory. These were kept at –20°C prior to the analysis and laboratory-scale fermentations.

2.2.3. Yeast strains

Three commercially available *S. cerevisiae* wine strains were used to perform grape juice fermentations: EC1118 (Lallemand, Montreal, Canada), AWRI 796 (Maurivin, Sydney, Australia) and VIN13 (Anchor, Montpellier, France).

2.2.4. Microvinification

Laboratory-scale fermentation (microvinification) was carried out as previously described by Pinu et al. (2012). In brief, the juice was chemically sterilised through the addition of 1000 µL of DMDC per L of juice and incubated at 25°C overnight, with stirring at 100 rpm. Pre-cultures were prepared by transferring a single colony freshly grown on YPD agar plates to 500-mL flasks, containing 100 mL of YPD broth (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L) and grown overnight at 28°C and at 150 rpm shaking. A concentration of 2.5 × 106 yeast cells/mL were added to a volume of 200 mL of chemically sterilised grape juice. The fermentation was carried out at 15°C under continuous agitation at 100 rpm in 250-mL Erlenmeyer flasks. In this study, we carried out two different batches of fermentation using three different wine yeast strains (EC1118, AWRI796 and VIN13). In the first experiment, fermentation of 2011 SB grape juice was

carried out in four replicates under two different experimental conditions: control (without linoleic acid supplementation, keeping the juice's natural linoleic acid content) and supplemented (linoleic acid level increased to 132 mg/L, based on Pinu et al. (2014a)). A further batch of fermentations was carried out in duplicate using a 2014 SB grape juice supplemented and not supplemented with d_8-3MH (1.5 µg/L).

2.2.5. Analysis of SB grape juice and wines

2.2.5.1. Winescan

The infrared (IR) spectra of the juice samples (~35 mL) were obtained prior to the fermentation and analysed with a Foss Winescan Flex instrument (Foss Electric, Denmark) using the factory-set calibrations for the following parameters: Brix, pH, TA, [citric acid], [glucose], [fructose], Folin C Index, OD_{280} and OD_{520}. The IR spectra of the wine samples (~35 mL) were obtained and analysed by a Foss Winescan Flex instrument, using the factory-set calibrations for the following parameters: pH, TA, [glucose], [fructose], [EtOH], [volatile acidity] and Folin C Index. Sample acquisition was carried out in duplicate with a cuvette backflush, sample pre-flush of 3 s, and an intake time of 7 s. An autoclean with Foss Winescan Cleaning agent (an aqueous hypochlorite solution) was carried out every 60 s. The areas of the spectra attributable to the absorption of water (PN400-445 and 770–940) were excluded from the analysed samples.

2.2.5.2. Gas chromatography–mass spectrometry

Analysis of volatile thiols in wines. Volatile thiols, including 3MH and 3MHA, were analysed according to the protocol described by Herbst-Johnstone et al. (2013b). In brief, the internal standards d2-3MHA (0.3 nmol) and d2-3MH (1.5 nmol), and the reagents butylated hydroxyanisole (2 mM) and ETP (100 mM), were added to 50 mL of wine. The pH was then adjusted to 10.0 ± 0.1, and the wine centrifuged to remove the precipitate. The supernatant was loaded onto an SPE cartridge, Supelclean ENVI-18 (6-mL cartridge

volume; 1-g sorbent; Supelco, Castle Hill, NSW, Australia). The analytes were recovered using 10 mL of dichloromethane and concentrated under nitrogen before being analysed by gas chromatography–mass spectrometry (GC–MS) using an Agilent 6890 N gas chromatograph (Santa Clara, CA, USA) equipped with a 5973 mass selective detector. The GC–MS settings are described by Herbst-Johnstone et al. (2013b). The absolute quantification of the varietal thiols was performed using calibration curves obtained from pure standards.

Analysis of other wine aroma compounds. Other aroma compounds of interest were analysed by headspace solid-phase microextraction (HS-SPME) and GC–MS, according to the protocol described by Herbst-Johnstone et al. (2013a). This method allowed us to determine the concentration of esters, higher alcohols, terpenes, C6-alcohols and volatile fatty acids. In short, 3.5 mg of NaCl, 50 µL of an ethanolic mixture containing 10 deuterated internal standards and a second standard mix, containing DL-3-octanol (499 µg/L), 4-decanol (1,005 µg/L) and 3,4-dimethylphenol (114 µg/L), were added to 10 mL of wine. The vial was then purged with argon and tightly capped before being incubated at 45°C while agitated at 700 rpm for 10 min. A DVB/CAR/PDMS fibre (SUPELCO, Bellefonte, PA, USA) was exposed into the capped vial, and after volatile extraction was transferred to the injection port of an Agilent 7890A GC System coupled to a mass selective detector model 5975C inert XL (Santa Clara, CA, USA), where the desorption of the analytes took place. The GC–MS settings are described by Herbst-Johnstone et al. (2013a). The absolute quantification of the analytes was performed using calibration curves of pure standards.

Non-volatile metabolites. MCF derivatization of the wine samples was carried out to determine the levels of amino and non-amino organic acids. The wine samples were prepared, according to the protocol described in Pinu et al. (2014a). Briefly, a wine sample of 500 µL was mixed with 20 µL of internal standard, 2,3,3,3-d_4-Lalanine-(10mM), and then lyophilised in a 12-L Labconco Freeze Dryer (Labconco Corporation, Kansas city,MO, USA). The sample was then resuspended in 200 µL of 1 M NaOH and mixed with 67 µL of

methanol and 34 µL of pyridine. Under continuous mixing, 20 µL of MCF was added. After 30 seconds, a second 20 µL aliquot of MCF was added. After a further 30 s, 400 µL of chloroformand 400 µL of 50 mM $NaHCO_3$ were added to the mixture. At this point, the sample was centrifuged, and the upper aqueous layer was discarded. The remaining sample was dried with anhydrous sodium sulphate before being transferred into a GC–MS vial. The derivatised wine samples were analysed according to the protocol described by Smart et al. (2010), using a gas chromatograph GC7890 (Agilent Technologies, USA) coupled to quadrupole mass spectrometer MSD 5975 (Agilent Technologies, USA). Trimethylsilyl (TMS) derivatisation was performed to determine the levels of sugars, and their derivatives using a sample preparation protocol described by Pinu et al. (2014a). Wine samples (20 µL) were mixed with 60 µL of methanol and 20 µL of the internal standard, D-ribitol (10 mM). The samples were dried with a rotary vacuum dryer (Thermo Fischer, Holbrock, NY, USA) and kept inside a desiccator overnight in order to eliminate all the water present in the samples. The dried wine samples were then resuspended in 80 µL of methoxyamine hydrochloride solution in pyridine (2 g/100 mL), and incubated at 30°C for 90 min. Following incubation, 80 µL of *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) was added, and the sample was further incubated at 37°C for 30 min. The sample was analysed by a gas chromatograph GC7890 (Agilent Technologies, USA) coupled to a quadrupole mass spectrometer MSD 5975 (Agilent Technologies, USA) according to the settings described by Villas-Bôas et al. (2006).

Data mining and statistical analysis. Automated mass spectral deconvolution and identification system (AMDIS) software was used for deconvoluting GC–MS chromatograms, and identifying metabolites using an in-house MS library. Identifications were based on both the MS spectrum of the derivatised metabolite and its respective chromatographic retention time. ChemStation (Agilent Technologies, Santa Clara, CA, USA) was used to determine the relative abundance of identified metabolites by using the GC base-peak value of a selected reference ion. These values were normalized by the values of the internal standards in each sample. The entire data mining, data normalisation

and ANOVA analyses were automated in R software as described in Smart et al. (2010) and Aggio et al. (2010). A students't-test was applied to determine whether the relative abundance of each identified metabolite was significantly different between wine making conditions (control fermentation versus fermentation of grape juices with increased linoleic acid). After log transforming the data, a principal component analysis (PCA) was performed using a web-based data analysis platform, MetaboAnalyst 3.0 (Xia et al., 2015).

2.3. Results and discussion

2.3.1. Effect of increased levels of linoleic acid on fermentation performance of different strains of *S. cerevisiae* and wine properties

The concentration of linoleic acid in the SB grape juice used in this study was 11 (±0.4) mg/L. Our results show that an increase in linoleic acid concentration to 132 mg/L in grape juice did not alter the duration of the fermentation nor any significant difference between the fermentation patterns and biomass yield when compared to the control wines (**Table 2.1**Error! Reference source not found. and **Figure 2.1**). However, the WineScan results showed that increase in the linoleic acid level affected an important wine parameter—the phenols (**Table 2.2**). Phenols are involved in the wine ageing acting as preservatives thanks to their antioxidant properties (Lopez-Velez et al., 2003; Minussi et al., 2003). The Folin C index measured by WineScan is an indicator of the phenol content of a sample. The addition of linoleic acid in grape juices resulted in the production of wines with 17%–39% lower Folin C index than their corresponding control wines ($p < 0.01$) (**Table 2.2**), which can be explained by the utilisation of these antioxidants by *S. cerevisiae* to overcome the apparent toxicity normally associated with linoleic acid (Ferreira et al., 2011).

Table 2.1. Concentration of *Saccharomyces cerevisiae* cells in Sauvignon blanc wines

Strain/treatment	Average cell number/mL	Standard deviation	*p-value*
EC1118 C	2.0×10^8	1.4×10^7	
EC1118 LA	2.1×10^8	2.8×10^7	0.908
AWRI796 C	2.8×10^8	1.6×10^7	
AWRI796 LA	3.1×10^8	3.0×10^7	0.248
VIN13 C	2.8×10^8	6.6×10^7	
VIN13 LA	3.2×10^8	4.6×10^7	0.551

C: control wines without linoleic acid supplementation; LA: wines derived from grape juices supplemented with linoleic acid.

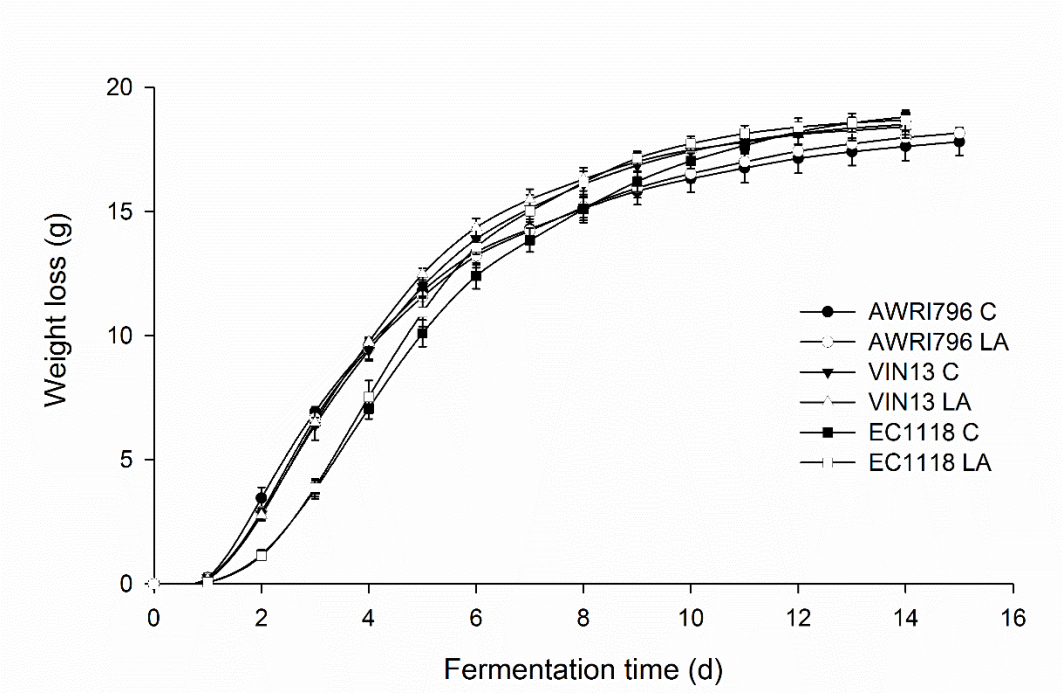

Figure 2.1. Weight loss of Sauvignon blanc juice fermented by three *Saccharomyces cerevisiae* strains. Fermentation was carried out in 250-mL flasks, containing 200 mL of juice (n=4) under 100 rpm shaking at 15°C. The three strains EC1118, AWRI796 and VIN13 fermented a control grape juice (C) and a juice with an increased linoleic acid concentration (LA).

2.3.2. Effect of the of linoleic acid on wine aroma compounds

The pre-fermentative addition of linoleic acid to the grape juice significantly affected the concentration of the varietal thiols in the resulting wines (**Table 2.3**), similarly to what was observed previously with *S. cerevisiae* EC1118 (Pinu et al., 2014a). For instance, an increased amount of linoleic acid caused a 100% reduction in 3MHA concentration ($p < 0.001$) when the juice was fermented by *S. cerevisiae* EC1118 compared to its corresponding control. The similar trend of 3MHA reduction, following linoleic acid supplementation of *S. cerevisiae* EC1118 ferments, was reported by Pinu et al. (2014a). Similarly, we observed a 69% and 72% reduction in 3MHA levels ($p < 0.001$) in wines fermented by *S. cerevisiae*, AWRI796 and VIN13, strains respectively. However, 3MH concentration did not show a consistent pattern of change in response to increased levels of linoleic acid for all three yeast strains tested. Wines produced by *S. cerevisiae*

EC1118 from the linoleic acid supplemented juices resulted in a 17% increase in 3MH concentration when compared to the control wines ($p < 0.001$), which was in agreement with Pinu et al. (2014b). On the other hand, *S. cerevisiae* AWRI796 strain showed an opposite trend with a 14% reduction in 3MH ($p< 0.05$) and *S. cerevisiae* VIN13 showed no significant change in 3MH levels ($p = 0.74$) when compared to the respective control wines (**Table 2.3**). Therefore, all the strains responded to the pre-fermentative addition of linoleic acid producing wines with reduced 3MHA levels and with a strain-specific response in relation to 3MH concentration.

Table 2.2. Oenological properties of Sauvignon blanc grape juice and wines analysed by WineScan.

Grape juice	pH	Total Acidity (g/L)	Brix (°)	Citric Acid (g/L)	Glucose (g/L)	Fructose (g/L)	FolinC Index
Sauvignon blanc juice 2011	3.2	9.0	19.3	0.7	96.7	93.6	7.9
Wines							
Saccharomyces cerevisiae strains	pH	Total Acidity (g/L)	Volatile Acidity (g/L)	Glucose (g/L)	Fructose (g/L)	FolinC Index	Ethanol v/v%
EC1118 C	3.0	7.6	0.2	0	0	11.6	13.0
EC1118 LA	3.0	7.6[b]	0.2	0	0	9.7[a]	12.9[b]
AWRI796 C	2.9	8.1	0.2	0	1.2	5.3	12.8
AWRI796 LA	2.9	8.2[b]	0.2[b]	0	1.7[b]	3.2[a]	12.7[b]
VIN13 C	2.9	7.8	0.1	0	0	8.4	13.0
VIN13 LA	2.9	7.9[b]	0.1	0	0	6.3[a]	12.9[b]

C: control wines without linoleic acid supplementation; LA: wines derived from grape juices supplemented with linoleic acid; [a]$p < 0.01$; [b]$p > 0.05$. n=4.

In addition to varietal thiols, the increase of linoleic acid in the grape juice also affected the production of important esters and other aroma compounds in the final wines (**Table 2.4**). Some of the compounds changed in concentration following similar patterns independent of the *S. cerevisiae* strain used, while others showed strain-specific patterns. We identified 30 aroma compounds in the wine samples including ethyl esters, acetate esters, higher alcohols, norisoprenoid terpenes, cinnamates, anthranilates and volatile fatty acids (**Table 2.4**). Among them, 12 out of 30 aroma compounds showed statistically significant changes ($p < 0.05$) in response to higher levels of linoleic acid, regardless of the

wine yeast strain. One such consistent response to linoleic acid was the reduction in the level of all acetate esters when compared to the control wines, which is in accordance with previous findings (Pinu et al., 2014a). A general reduction of the acetylation process in the presence of linoleic acid was observed in previous research conducted on beer and wine fermentation (Pinu et al., 2014a; Thurston et al., 1981; Thurston et al., 1982). The acetate esters are synthesised by an alcohol acetyltransferase (AATase) that catalyse the reaction between an alcohol and acetyl coenzyme A (Malcoprs et al., 1991; Malcorps & Dufour, 1992; Minetoki et al., 1993; Yoshioka & Hashimoto, 1981, 1983). It was noticed previously that expression of the AATase gene, ATF1, is largely repressed by the supplementation of unsaturated fatty acids to the wort (Fujiwara et al., 1999; Fujiwara et al., 1998; Moonjai et al., 2003). This AATase is responsible for the acetylation of many other compounds, including 3MH, which is converted into 3MHA (Swiegers et al., 2007). Therefore, although we did not analyse the expression of ATF1, we assume that independent of the *S. cerevisiae* strain, linoleic acid may repress ATF1 expression in the cells, resulting in a decreased production of many acetylated aroma compounds. Further, transcriptomics analysis is needed to confirm the ATF1 repression by linoleic acid under our fermentation conditions.

In addition to the reduced amount of acetate esters produced in response to increased levels of linoleic acid, we observed an increase in the levels of five higher alcohols in wines produced from the juices fermented by *S. cerevisiae* EC1118 and VIN13 strains and two higher alcohols in wines produced by AWRI796 (**Table 2.4**). The possible reduction in AATase catalysing the synthesis of acetate esters from alcohols and acetyl-CoA due to linoleic acid repression supports the observed accumulation of these higher alcohols, which are precursors for acetate ester synthesis (Moonjai et al., 2003). The increased concentration of linoleic acid in the grape juice also affected other wine aroma compounds, such as ethyl esters, norisoprenoid terpenes, C6 compounds, fatty acids, cinnamates and anthranilates, displaying strain-specific patterns (**Table 2.4**). However, the

patterns do not seem to have a specific trend, and thus, the mechanisms behind the effect of linoleic acid on these compounds remains unclear.

Table 2.3. Concentration of varietal thiols in Sauvignon blanc fermented from grape juice supplemented and non-supplemented with linoleic acid.

Saccharomyces cerevisiae strains	3MHA	3MH
	ng/L (n=4)	ng/L (n=4)
EC1118 C	107 ± 7	741 ± 13
EC1118 LA	ULOD[b]	866 ± 23[b]
AWRI796 C	124 ± 4	803 ± 17
AWRI796 LA	39 ± 4[b]	690 ± 86[a]
VIN13 C	149 ± 7	1241 ± 17
VIN13 LA	41 ± 3[b]	1234 ± 38[c]

C: control wines without linoleic acid supplementation; LA: wines derived from grape juices supplemented with linoleic acid; ULOD = under limit of detection; [a]$p < 0.05$; [b]$p < 0.001$; [c]$p > 0.05$.

2.3.3. Effect of the increased linoleic acid concentration on other wine metabolites

Wine is a complex matrix that contains thousands of metabolites in addition to several hundred aroma compounds (Ebeler & Thorngate, 2009; Roland et al., 2012). In our study, we detected more than 200 non-volatile metabolites by GC–MS and 53 of them were accurately identified using our in-house MS library. Similarly to the volatile compounds, the level of many non-volatile metabolites in the wine changed in response to higher levels of linoleic acid (**Figure 2.2**). We carried out principle component analysis (PCA) using 33 metabolites whose levels changed significantly in response to the linoleic acid increment ($p < 0.05$) (**Figure 2.2**), showing clearly that the metabolite profiles of the wines obtained from the grape juices with higher linoleic acid level were completely different from control wines. Once again, some of the changes in the levels of non-volatile metabolites were strain-specific, whereas others were similar among all three strains.

All strains responded to linoleic acid by producing wines with a lower level of nine proteinogenic amino acids (alanine, aspartic acid, glutamic acid, isoleucine, leucine, serine, threonine, tryptophan and tyrosine; all $p < 0.01$), which are preferred sources of nitrogen for *S. cerevisiae* under winemaking conditions (Pinu et al., 2014b). Thus, we can assume that a pre-fermentative increase in linoleic acid also induced the wine yeasts to consume more of these amino acids compared to the control wines. However, we did not observe a significant change in biomass yield in response to linoleic acid. Among the non-proteinogenic amino acids, 4-aminobutyric acid (GABA) ($p < 0.0001$) also showed a drastic decrease in levels in response to linoleic acid. It is possible to relate the reduction in GABA levels in the wine to the lower antioxidant content of the wines determined by WineScan analysis. Previous studies showed that when *S. cerevisiae* is exposed to anoxia, it undergoes a transient oxidative stress (Coleman et al., 2001) and high sugar-containing media, like the grape juice, increase ROS production under anaerobiosis (Landolfo et al., 2008). Under oxidative stress, *S. cerevisiae* stores a large proportion of GABA in the cell (Bach et al., 2009), and GABA metabolism plays a fundamental role in yeast stress tolerance (Coleman et al., 2001). Due to its role in lipid peroxidation (Ferreira et al., 2011), linoleic acid supplementation to the grape juice represents an additional oxidative stress for the yeast compared to the control. Therefore, we can infer that yeast uptakes more GABA, reducing its level in the wine in order to overcome the toxicity of linoleic acid.

Table 2.4. Concentration of esters and other aroma compounds in Sauvignon blanc wine produced from grape juice supplemented and non-supplemented with linoleic acid.

Compounds	*Saccharomyces cerevisiae* strain					
	EC1118		AWRI796		VIN13	
	Control	LA	Control	LA	Control	LA
	µg/L					
Ethyl esters						
Ethyl isobutyrate	19 ± 1	24 ± 1^{c}	33 ± 4	29 ± 3^{d}	25 ± 2	26 ± 3^{d}
Ethyl butanoate	247 ± 21	162 ± 7^{c}	297 ± 12	309± 18^{d}	241 ± 3	198 ± 5^{c}
Ethyl 2-methyl butanoate	2.6 ± 0.2	3.2 ± 0.1^{a}	3.1 ± 0.3	3.0 ± 0.2^{d}	3.2 ± 0.1	3.7 ± 0.1^{b}
Ethyl isovalerate	3.8 ± 0.4	4.1 ± 0.1^{d}	4.1 ± 0.4	3.9 ± 0.3^{d}	4.5 ± 0.4	5.1 ± 0.3^{a}
Ethyl hexanoate	712 ± 58	452 ± 31^{c}	620 ± 18	528 ± 55^{a}	563 ± 5	475 ± 39^{a}
Ethyl heptanoate	4.0 ± 0.01	4.0 ± 0.0^{b}	4.0 ± 0.0	4.3 ± 0.2^{d}	4.3 ± 0.0	4.8 ± 0.2^{b}
Ethyl octanoate	714 ± 96	930 ± 46^{a}	619 ± 84	872 ± 137^{a}	719 ± 74	792 ± 146^{d}
Ethyl decanoate	341 ± 19	614 ± 70^{b}	268 ± 34	609 ± 129^{a}	466 ± 47	526 ± 126^{d}
Ethyl dodecanoate	27 ± 3	131 ± 32^{b}	18 ± 4	104 ± 19^{b}	16 ± 1	84 ± 15^{b}
Acetate esters						
Isoamyl acetate	2941 ± 255	777 ± 21^{c}	3695 ± 223	2478 ± 220^{b}	3132 ± 75	1747 ± 104^{c}
Hexylacetate	116 ± 8	28 ± 3^{c}	123 ± 6	92 ± 13^{a}	110 ± 3	56 ± 14^{b}
Cis-3-hexenyl acetate	6.6 ± 0.4	2.8 ± 0.1^{c}	7.7 ± 0.4	4.8 ± 0.1^{b}	7.3 ± 0.1	3.7 ± 0.1^{c}
β-phenylethyl acetate	449 ± 10	182 ± 7^{c}	342 ± 17	183 ± 3^{c}	260 ± 5	129 ± 5^{c}
Other esters						
Methyl octanoate	4.2 ± 0.3	3.9 ± 0.4^{d}	3.9 ± 0.4	3.6 ± 0.3^{d}	4.0 ± 0.4	3.5 ± 0.5^{d}
Ethyl-(L)-lactate	2944 ± 170	3753 ± 180^{a}	2611 ± 347	2778 ± 429^{d}	2523 ± 314	2702 ± 303^{d}
Diethyl succinate	112 ± 2	125 ± 4^{a}	121 ± 2	122 ± 3^{d}	128 ± 1	133 ± 3^{d}
Higher alcohol						
Isobutanol	3113 ± 521	6601 ± 826^{b}	5623 ± 902	3320 ± 1204^{d}	4093 ± 982	6099 ± 613^{b}
Isoamyl alcohol	117852 ± 6358	148409 ± 6561^{c}	122895 ± 1244	155638 ± 3776^{c}	130879 ± 8148	168416 ± 5505^{c}

Benzaldehyde	1.7 ± 0.1	7.9 ± 1.1[b]	2.4 ± 0.3	2.6 ± 0.0[d]	1.5 ± 0.2	3.8 ± 0.6[b]
Benzyl alcohol	53 ± 0.5	66 ± 2[c]	58 ± 1	58 ± 1[d]	53 ± 0.4	61 ± 3[a]
Phenylethyl alcohol	26162 ± 945	31555 ± 1759[b]	22228 ± 248	24324 ± 419[c]	18124 ± 1031	22124 ± 574[b]
Norisoprenoid terpenes						
β-damascenone	3 ± 0.2	2 ± 0.1[c]	3 ± 0.2	2 ± 0.1[c]	3 ± 0.1	2 ± 0.1[c]
(+)-terpinen-4-ol	1.6 ± 0.04	2.0 ± 0.3[d]	1.8 ± 0.1	1.6 ± 0.1[a]	1.4 ± 0.0	1.7 ± 0.4[d]
α-terpineol	2.6 ± 0.1	2.4 ± 0.1[a]	3.4 ± 0.2	2.5 ± 0.7[d]	3.2 ± 0.2	2.7 ± 0.7[d]
β-citronellol	86 ± 5	131 ± 13[b]	67 ± 11	103 ± 5[b]	112 ± 10	92 ± 7[a]
C6 compounds						
Hexanol	1369 ± 28	2028 ± 157[b]	1424 ± 17	1728 ± 102[b]	1344 ± 28	1679 ± 130[a]
Fatty acids						
Isobutyric acid	743 ± 34	805 ± 31[a]	911 ± 74	1019 ± 65[d]	747 ± 21	861 ± 41[b]
Hexanoic acid	5335 ± 237	4560 ± 139[b]	4354 ± 163	4450 ± 113[d]	4264 ± 132	4030 ± 209[d]
Octanoic acid	3795 ± 220	2210 ± 225[c]	2999 ± 424	2601 ± 164[a]	3028 ± 494	2062 ± 266[a]
Cinnamates and anthranilates						
Ethyl (di)hydrocinnamate	0.7 ± 0.0	0.7 ± 0.2[d]	0.4 ± 0.0	0.4 ± 0.0[d]	0.8 ± 0.0	0.8 ± 0.1[a]

C: control wines without linoleic acid supplementation; LA: wines derived from grape juices supplemented with linoleic acid; [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$; [d]$p > 0.05$.

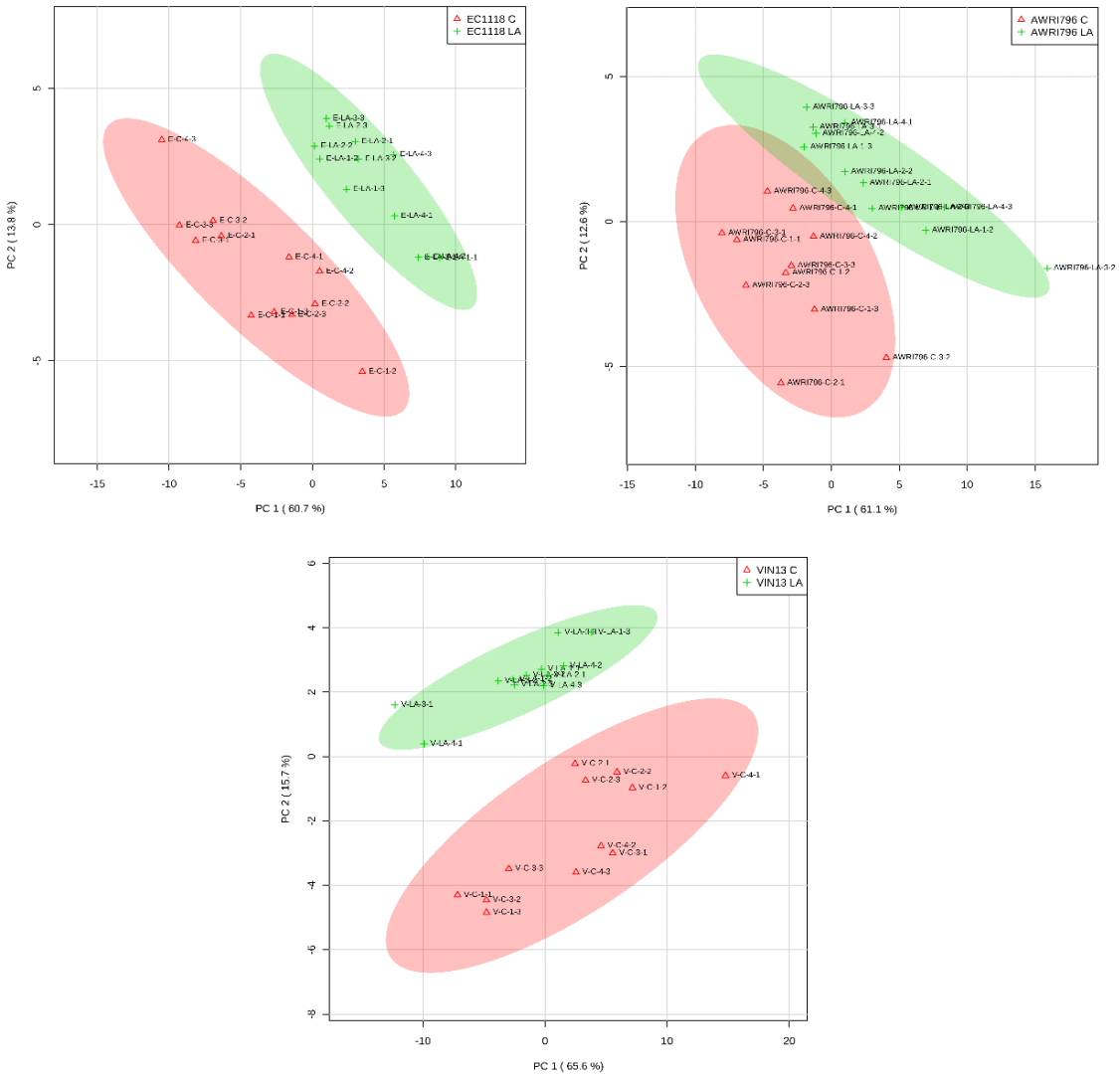

Figure 2.2. Two-dimensional projections of principal component analysis (PCA) using 33 statistically significant identified non-volatile metabolites. The three *Saccharomyces cerevisiae* wine strains EC1118, AWRI796 and VIN13 fermented a control Sauvignon blanc grape juice (C) and a linoleic acid supplemented juice (LA).

On the other hand, the level of decanoic acid increased considerably ($p < 0.001$) in wines fermented by both *S. cerevisiae* EC1118 and VIN13. The release in the wine of a toxic compound such as decanoic acid (Liu et al., 2013) could have been facilitated by an increase in yeast membrane fluidity when linoleic acid concentration increased, thanks to higher availability of this unsaturated fatty acid in grape juice (Watson & Rose, 1980).

Interestingly, wines fermented by *S. cerevisiae,* AWRI796, contained a higher level of the amino acid cysteine in response to increased level of the linoleic acid in the juice (p = 0.02). Cysteine is a sulfur source for microorganisms including *S. cerevisiae* and can be utilised to form volatile sulfur compounds and thiols (Bonnarme et al., 2000; Dainty et al., 1989; Morales et al., 2005; Russell et al., 1995; Seefeldt & Weimer, 2000). In our case, AWRI796 responded to linoleic acid by accumulating more cysteine in the wine and, at the same time, reducing the levels of the varietal thiol 3MH. Although it has been reported that higher levels of cysteine increases the concentration of both 3MH and 3MHA in wines fermented by *S. cerevisiae* EC1118 (Harsch & Gardner, 2013), our results showed a clear negative correlation between 3MH and cysteine using *S. cerevisiae* AWRI796. Since one of the main features of AWRI796 is the production of low levels of aroma compounds (www.maurivin.com), an accumulation of a precursor such as cysteine is not surprising.

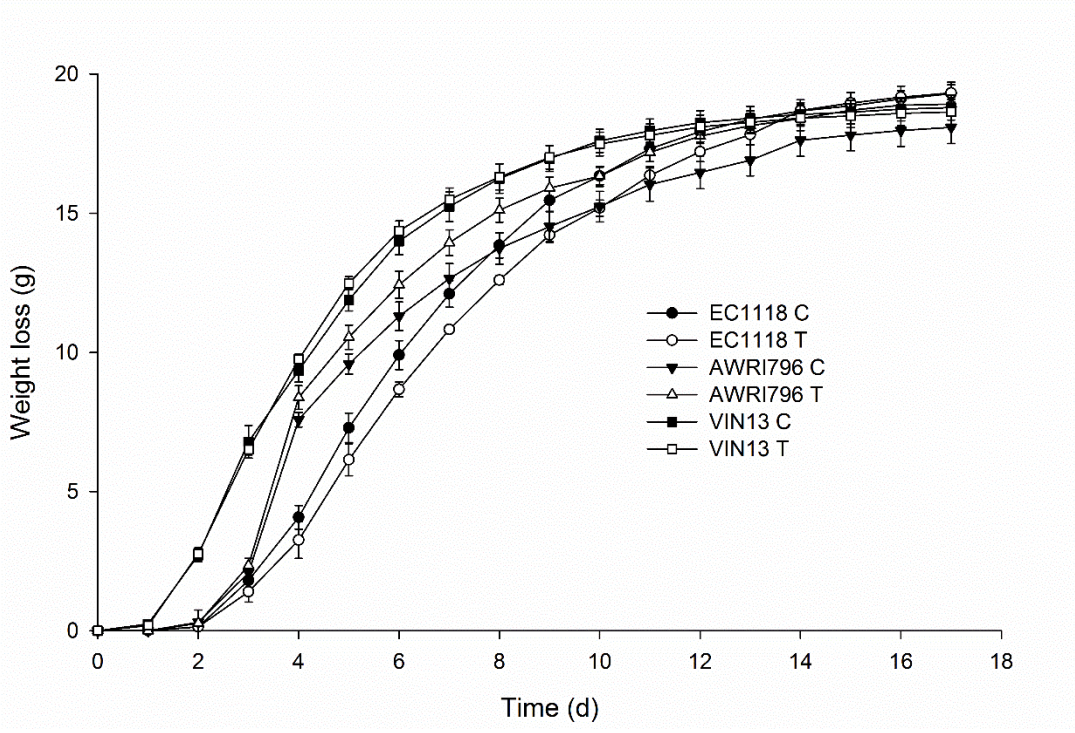

Figure 2.3. Weight loss of Sauvignon blanc juice fermented by three *Saccharomyces cerevisiae* strains. Fermentation was performed in 250-mL flasks, containing 200 mL of juice (n = 4) under 100 rpm shaking at 15°C. The three strains EC1118, AWRI796 and VIN13 fermented a control grape juice (C) and a juice supplemented with a deuterated analogue of 3MH (T).

2.3.4. Effect of the 3MH grape juice supplementation on the wine varietal thiols

Since the addition of linoleic acid in grape juice resulted in an increased production of 3MH in the wines produced by *S. cerevisiae* EC1118, we expected that linoleic acid inhibition of 3MH acetylation (3MHA production) would result in an obvious accumulation of 3MH for all three strains, as observed for the EC1118 strain. However, the other two yeast strains tested did not show this pattern despite showing a decreased level of 3MHA in the final wines. We therefore hypothesised that 3MH could exert a feedback inhibition of its own production in some *S. cerevisiae* strains, as observed for other microbial biosynthetic products such as amino acids and proteins (Chubukov et al., 2014). We carried out another experiment where we fermented SB grape juice, supplemented with a deuterated isotope of 3MH (d_8-3MH) using the three different *S. cerevisiae* strains, EC1118, AWRI796 and VIN13. We measured the two varietal thiols produced at the end of the fermentation to verify if 3MH exerts feedback regulation or not. The results of the fermentation performance showed that the addition of d_8-3MH to the grape juice did not affect the growth of any of the *S. cerevisiae* strains tested (**Figure 2.3**). 3MH supplementation resulted in an average 10% decrease in the *de novo* (non-isotopically labelled) 3MH concentration in wines fermented by EC1118, whereas a 14% increase occurred in the *de novo* 3MH level in wines fermented by AWRI796 strain (**Table 2.5**). Wines fermented by VIN13 did not show significant change in 3MH level in response to d_8-3MH juice supplementation. On the other hand, d_8-3MH juice supplementation did not affect the total level of thiols (3MH + 3MHA) in wines fermented by *S. cerevisiae* EC1118 and VIN13, but wines fermented by AWRI796 showed an increase of 15% in the total amount of thiols (3MH + 3MHA) in response to 3MH juice supplementation. Therefore, only EC1118 responded to d_8-3MH supplementation with a negative feedback regulation pattern, which was surprising. AWRI796 increased the production of both 3MH and 3MHA, whereas VIN13 production of varietal thiols was unaffected. Thus, the regulation of 3MH and 3MHA production during wine fermentation seems to be strain-specific. Whilst a

feedback regulation exhibited by *S. cerevisiae* EC1118 would explain the poor correlation between varietal thiol levels and thiol precursor availability in the juice (Pinu et al., 2012), the mechanism behind AWRI796 positive feedback regulation of thiol biosynthesis is still puzzling, and reinforces the critical importance of choosing the most suitable wine yeast strain to obtain a wine with the desired aroma and flavour profile.

Table 2.5. Varietal thiol concentrations in Sauvignon blanc wines fermented from grape juice supplemented and non-supplemented with d_8-3MH.

		Average concentration (n=4)				
		3MHA	3MH	d_8-3MHA	d_8-3MH	3MHA+3MH
Strain	Condition	ng/L				nM
EC1118						
	C	2731 ± 172	13619 ± 834			117 ± 7
	M	3710 ± 622[a]	12361 ± 157[a]	90 ± 19	130 ± 1	113 ± 3[c]
AWRI796						
	C	2466 ± 646	12700 ± 694			108 ± 7
	M	2991 ± 127[c]	14887 ± 925[b]	86 ± 7	173 ± 19	128 ± 8[a]
VIN13						
	C	619 ± 36	10006 ± 181			78 ± 1
	M	653 ± 53[c]	10241 ± 362[c]	50 ± 12	237 ± 45	80 ± 3[c]

3MH: 3-mercaptohexanol; 3MHA: 3-mercaptohexyl acetate; C: control wines; T: wines fermented from d_8-3MH supplemented juices; [a]$p < 0.05$; [b]$p < 0.01$; [c]$p > 0.05$.

2.4. Acknowledgements

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CHAPTER THREE

Metabolomics reveals the effect of linoleic acid on *Saccharomyces cerevisiae* metabolism

Francesca Casu[1], Farhana R. Pinu[2], Eliezer Stefanello[3], David R. Greenwood[1,2] and Silas G. Villas-Bôas[1]

[1] School of Biological Sciences, the University of Auckland, 3A Symonds Street, Auckland 1010, New Zealand,

[2] The New Zealand Institute for Plant and Food Research Limited, 120 Mt Albert Rd, Sandringham, Auckland 1025, New Zealand

[3] Department of Biochemistry, Institute of Chemistry, University of São Paulo, Av. Prof. Lineu Prestes, 748, São Paulo, SP, Brazil

The content of this chapter has not been published

3.1. Introduction

Fermented foods such as bread, wine, beer and cheese have been part of human history for millennia (McGovern et al., 2003; McGovern et al., 2004). The fermentation is performed using fungi and/or bacteria, which are involved in the production of a large range of products. Among the fungi, many yeasts can ferment (Van Dijken et al., 1986), but only a few can grow in naturally harsh media such as grape juice. *Saccharomyces cerevisiae* is one yeast that evolved to grow in media with high sugars, low nitrogen and low pH over thousands of years (Piškur et al., 2006). However, there are also a few limiting factors for *S. cerevisiae* growth under anaerobic conditions such as the availability of trace amounts of fatty acids required for maintenance of the cell membrane (Ferreira et al., 2011). Moreover, *S. cerevisiae* is unable to synthesise unsaturated fatty acids in the absence of molecular oxygen (Hiltunen et al., 2003). Among the unsaturated fatty acids, linoleic acid, is known to affect the aroma properties of different fermented beverages including beer (Moonjai et al., 2003) and wine (Casu et al., 2016; Pinu et al., 2014b).

The metabolism of *S. cerevisiae* is significantly affected by linoleic acid under anaerobic conditions (Casu et al., 2016; Pinu et al., 2014b). The supplementation of linoleic acid alters the production of different aroma compounds including volatile thiols and esters by *S. cerevisiae,* indicating a clear effect on yeast metabolism (Casu et al., 2016). However, it is unknown whether the central carbon metabolism and the energetics of the cell are affected by the availability and subsequent absorption of this fatty acid under anaerobic conditions. Previously, it was observed that linoleic acid is absorbed by yeast cells and incorporated into membrane lipids under both aerobic and anaerobic conditions (Avery et al., 1996; Ferreira et al., 2011; Moonjai et al., 2003) Therefore, it is clear that it is able to enter the cell. After incorporation, linoleic acid degradation in *S. cerevisiae* cells under aerobic conditions is comparatively well-studied (Hiltunen et al., 2003; Lehninger et al., 2005). The presence of oxygen allows unsaturated fatty acids to be transformed into shorter chain fatty acids and consequently degraded through the β-oxidation pathway

which, in *S. cerevisiae* cells, takes place exclusively in the peroxisomes (Hiltunen et al., 2003; Lehninger et al., 2005).

In this study, the effect of different linoleic acid isomers at different concentrations on *S. cerevisiae* metabolism was investigated under aerobic and anaerobic conditions using metabolomics and isotope-labelling experiments.

3.2. Material and methods

3.2.1. Chemicals

All chemicals used in this study were of analytical grade. Chloroform, absolute ethanol (≥99.5%), magnesium sulfate, potassium phosphate, zinc sulfate, copper sulfate, calcium chloride, sodium chloride, ethyl acetate and boric acid were obtained from Merck (Darmstadt, Germany). Linoleic acid (∆9Z,12Z-LA), conjugated linoleic acid (∆9Z,11E-CLA) and linoleic acid (Δ9Z,12Z)-$^{13}C_{18}$ were purchased from Nu Chek Prep. Inc (Elysian, MN, USA). Methanol-d_4 was obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Methanol, anhydrous sodium sulfate, pyridine, 2,3,3,3-d_4-alanine, D-ribitol, methyl chloroformate (MCF) and N-methyl-N-(trimethylsilyl)-trifluoroamide (MSTFA, derivatization grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methoxyamine hydrochloride was purchased from Fluka (Steinheim, Switzerland). Grade 1 water 77 (BARNSTEAD® NANOpure DIamond™ Water Purification System, Waltham, MA, USA) was used to prepare most of the solutions.

3.2.2. Yeast strain

The yeast strain used for all fermentations was *Saccharomyces cerevisiae* EC1118 (Lallemand, Montreal, Canada), which was maintained on YPD agar medium (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L, agar 20 g/L) at 30°C.

3.2.3. Flask culture

S. cerevisiae was cultivated in shake flasks (n = 3) with 200 mL of medium containing D-glucose (20 g/L), $(NH_4)_2SO_4$ (5 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), KH_2PO_4 (3 g/L), vitamins and trace metals (Verduyn et al., 1992). The cultures were incubated at 30°C and 150 rpm. Aerobic cultivations were carried out in baffled flasks (500 mL, Schott, Mainz, Germany) to promote better aeration than the regular flasks. Anaerobic cultivations were performed using flasks sealed with rubber stoppers and home brew locks filled with 3 mL

of sterilised distilled water to prevent the oxygen entry. The anaerobic flasks were supplemented with 10 mg/L ergosterol and they were flushed with nitrogen prior to cultivation. Pre-inocula were prepared in 500 mL baffled flasks aerobically from which cells in exponential growth were used to inoculate the experimental flasks with an initial OD_{600nm} of 0.02. Fermentations were performed with culture media either supplemented or not with one of two isomers of linoleic acid at two different concentrations (**Table 3.1**). The sampling was carried out when the cells reached 2.5 OD_{600nm}, the OD_{600nm} at which the yeast cells in all growth conditions were at exponential phase.

Table 3.1. Concentrations of linoleic acid isomers in minimal mineral media used to culture *Saccharomyces cerevisiae* EC1118 under different experimental conditions.

Condition	Isomer	Concentration (mg/L)
Anaerobic		
Ana C	-	-
Ana LA1	9Z,12Z-Linoleic acid	66
Ana LA2	9Z,12Z -Linoleic acid	132
Ana CLA1	9Z,11E-Linoleic acid	66
Ana CLA2	9Z,11E-Linoleic acid	132
Ana 13LA1	9Z,12Z-Linoleic acid-$^{13}C_{18}$	66
Aerobic		
Aer C	-	-
Aer LA1	9Z,12Z-Linoleic acid	66
Aer 13LA1	9Z,12Z-Linoleic acid-$^{13}C_{18}$	66

3.2.4. Sampling and extraction of intracellular metabolites

The intracellular metabolites were sampled, quenched and extracted according to our lab's previous published protocol (Smart et al., 2010). In brief, aliquots of 50 mL of broth were rapidly transferred to a cold glycerol-saline solution (3:2 (vol/vol)) to quench cell metabolism. After 5 minutes of acclimatisation at -23°C, the cells were spun down at 36,086*g* for 20 minutes at -20°C. The cell pellet was resuspended in 2 mL of cold washing

solution (glycerol-saline 1:1) and recentrifuged at 36,086*g* for 20 minutes at -20°C. The supernatant was discarded and 20 µL of 2,3,3,3-d_4-alanine (internal standard) was added to the cell pellet before extraction of intracellular metabolites. A methanol-water (50% (v/v), -20°C) solution (2.5 mL) was added to the sample, which was mixed vigorously with a vortex mixer, and frozen at -80°C. At this point, three freeze/thaw cycles followed in order to release the intracellular metabolites into the extracting solution. Each sample was thawed in an ice bath (≈4°C) and mixed vigorously before being frozen again at -80°C. After the third thawing, the sample was centrifuged at 20,800*g* for 15 minutes at -20°C and the supernatant collected and stored at -80°C. Another 2.5 mL of cold methanol-water (70% (v/v), -20°C) solution was added to cell pellet, mixed and centrifuged at 20,800*g* for 15 minutes at -20°C. This supernatant was then collected and pooled together with the supernatant previously obtained. The extracted metabolites were stored at -80°C and freeze-dried using a 12 L Labconco Freeze Dryer (Labconco Corporation, Kansas city, MO, USA) before derivatisation. The cell pellet was used for biomass quantification. The pelleted cells were resuspended in 10 mL of bidistilled water and filtered under vacuum on a pre-weighted membrane filter (0.2-µm pore, 47 mm). The filter was dried for 2 min in a microwave oven and kept in a desiccator overnight before reweighing.

3.2.5. Extracellular metabolites

Spent culture medium (10 mL) was filtered using 0.2-µm pore membrane filter. The spent culture was then divided into 3 aliquots of 2 mL and 20 µL of the internal standard (2,3,3,3-d_4-alanine) was added to each of them prior to storage at -80°C. Before derivatisation the samples containing extracellular metabolites were freeze-dried using a 12 L Labconco Freeze Dryer (Labconco Corporation, Kansas city, MO, USA). The remaining 4 mL of spent culture was stored at -80°C until TMS derivatisation and ethanol quantification analysis.

3.2.6. Gas chromatography–mass spectrometry

3.2.6.1. Methylchloroformate (MCF) derivatisation

The protocol described by Smart and co-workers (Smart et al., 2010) was used to derivatise non-volatile compounds such as amino and non-amino organic acids, some primary amines and alcohols in both extracellular and intracellular samples. In short, the freeze-dried samples were resuspended in 200 µL of 1 M NaOH, mixed with 67 µL of methanol and 34 µL of pyridine. An amount of 20 µL of MCF was added and mixed for 30s using a vortex mixer. Then another aliquot of 20 µL of MCF was added and mixed again for 30 s. After this, 400 µL of chloroform was added to the samples and mixed for 10 s followed by the addition of 400 µL of 50 mM $NaHCO_3$ and a further 10 s vortexing. The samples were then centrifuged and the upper layer was discarded. Anhydrous sodium sulfate was added to absorb the remaining water in the samples. The dried samples were transferred to GC-MS vials and analysed by a gas chromatograph GC7890 (Agilent, Santa Clara, CA, USA) coupled to a quadrupole mass spectrometer MSD 5975 (Agilent, Santa Clara, CA, USA) operated at 70 eV. The column used was a Zebron ZB-1701 (Phenomenex), 30 m x 250 µm (internal diameter) x 0.15 µm (film thickness), with a 5 m guard column. The sample (1 µL) was injected under pulsed splitless mode (1.8 bar He for 1 min, 20 ml min^{-1} split flow after 1.01 min). The gas flow into the column was held constant at 1.0 mL of He per min. The temperature program of the GC oven is shown in **Table 3.2**. The temperatures of the inlet, interface and quadrupole were 290°C, 250°C and 200°C, respectively. Before each injection the column of the GC was equilibrated for 6 min. The mass spectrometer was operated in scan mode and data collected after 5 min with a mass range of 38-650 amu at 1.47 scans per s.

Table 3.2. Settings of the GC oven for the analysis of MCF derivatised samples.

Oven stage	°C/min	Rise of the temperature (°C)	Hold (min)
Initial	-	45	2
1	9	180	5
2	40	220	5
3	40	240	11.5
4	40	280	2

3.2.6.2. Trimethyl silyl (TMS) derivatisation

Sugar and their derivatives were only analysed in the extracellular samples using the TMS derivatisation method as described in Pinu et al. (2014b). In summary, 60 µL of methanol and 20 µL of the internal standard D-ribitol (10mM), were added to 20 µL of spent culture medium and then dried using a rotary vacuum dryer (Thermo Fischer, Holbrock, NY, USA). The dried samples were resuspended in 80 µL of methoxyamine hydrochloride solution in pyridine and incubated at 30°C for 90 minutes. Then 80 µL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added and the samples were incubated again at 37 °C for 30 minutes. After derivatisation, the samples were analysed using the gas chromatograph and column described above. The settings of the GC-MS are described in Villas-Bôas et al. (2006). Briefly, the sample (1 µL) was injected in pulsed split mode with a split ratio of 25:1 and a split flow at 32.934 mL/min. The He gas flow through the column was 1.3174 mL/min. The temperature program of the GC oven is shown in **Table 3.3**. The temperatures of the inlet, interface and quadrupole were 230°C, 300°C and 200°C, respectively. The mass spectrometer was operated in scan mode with a mass range of 40-650 amu at 1.47 scans per s.

Table 3.3. Settings of the GC oven for the analysis of TMS derivatised samples.

Oven stage	°C/min	Rise of the temperature (°C)	Hold (min)
Initial	-	70	5
1	10	179	-
2	0.5	180	2
3	10	220	1
4	2.5	265	1
5	10	280	1
6	1	290	0.6

3.2.6.3. Ethanol quantification

Quantification of ethanol was carried out in the spent culture medium samples using an in house GC-MS method. In summary, 100 µL of sample was mixed with 10 µL of internal standard, d_4-methanol, in a 2.5 mL safe lock Eppendorf tube. Approximately 50-100 mg of NaCl was added to each sample (ensuring complete saturation) and vortexed for 20 seconds. Then, 500 µL ethyl acetate were added and mixed for 1 min using a vortex mixer and then centrifuged at 3220 *g* for 3 minutes in a benchtop centrifuge (Eppendorf, Hamburg, Germany). The organic phase was collected and analysed in the GC-MS system as above. The sample was injected to the GC under split mode at a 100:1 split ratio under constant flow of 48.851 mL/min. The inlet temperature was kept at 180°C. Initially, the GC oven temperature was held at 50°C for 1 min, and then raised to 200°C at 40 °C/min. The total running time was 4.75 min. The interface and quadruple temperatures were 230°C and 150°C, respectively. The MS detector was turned off between 2.03 min to 2.21 min to offload the ethyl acetate peak. The ions (*m/z*) used for identification were: D_4-methanol (**33**, 35) and ethanol (**31**, 45). The ions shown in bold were used for the quantification of the ethanol using a five point calibration curve of pure ethanol standard in water solutions (1.6-23.7 g/L).

3.2.7. Data analysis

The raw data from the GC-MS were automatically analysed by the Automated Mass Spectral Deconvolution and Identification software program (AMDIS). Identification was performed using both the MS spectrum of the metabolite and its respective retention time using an in-house MS library. Raw data were then transformed into AIA format (.cdf) files using ChemStation (Agilent Technologies, Santa Clara, CA, USA). The .cdf files were used for normalisation, ANOVA analysis and data mining using an automated in-house R software as described in Smart et al. (2010) and Aggio et al. (2010). The relative abundance of identified metabolites was calculated based on the maximum height of the selected reference ion for the compound peaks. These values were then normalised by the biomass and the internal standard (2,3,3,3-d_4-alanine). In addition, for the extracellular analysis, the medium composition was subtracted from the sample to determine relative secretion and uptake. A students' t-test was performed to determine the statistical significance ($p < 0.05$) between different experimental conditions. The profile of metabolites identified in intracellular samples was used to perform a pathway analysis using a R software package called Pathway Activity Profiling (PAPi) (Aggio et al., 2010). PAPi is a bioinformatic tool that helps to generate hypotheses and to support experimental findings correlating the intracellular metabolite levels using the KEGG pathway database (http://www.kegg.jp/). An activity score is assigned to the pathways which are potentially active suggesting whether they are up- or down-regulated when different experimental conditions are compared. Only pathways with a significant activity score were considered using a *p*-value of 0.05.

3.3. Results

3.3.1. Linoleic acid affects the fermentation performance

The effect of LA and CLA supplementation on the fermentation performance of *S. cerevisiae* was determined by using two key fermentation parameters, maximum biomass production and maximum growth rate. **Figure 3.1** presents the growth curves of *S. cerevisiae* under the different experimental conditions tested. It was observed that both LA and CLA supplementation significantly reduced the yeast growth rate up to 57% and the biomass titre up to 29% ($p < 0.05$) under both aerobic and anaerobic condition compared to the control cultures (non-supplemented media) in a dose-dependent way (**Figure 3.1** and **Table 3.4**). These results were in accordance with Ferreira et al. (2011) who also showed that LA can reduce the growth rate of *S. cerevisiae* cells. Interestingly, the higher the LA and CLA concentration the lower the biomass titter and growth rate were observed, suggesting a dose-dependent effect.

Table 3.4. Maximum biomass production and growth rate of *Saccharomyces cerevisiae* EC1118 grown on glucose in the presence and absence of different concentrations of linoleic acid isomers.

Condition	Biomass OD_{600nm}	Growth rate OD_{600nm} / h
Anaerobic		
C	4.98 ± 0.21	0.60 ± 0.02
LA1	4.30 ± 0.21[a]	0.47 ± 0.02[a]
LA2	4.25 ± 0.22[a]	0.40 ± 0.01[a]
CLA1	3.71 ± 0.25[a]	0.41 ± 0.02[a]
CLA2	3.52 ± 0.10[b]	0.26 ± 0.02[b]
Aerobic		
C	5.66 ± 0.15	0.92 ± 0.01
LA1	4.34 ± 0.12[b]	0.80 ± 0.01[a]

C = control; LA = linoleic acid supplementation, CLA = conjugated linoleic acid supplementation; 1 = 66 mg/L; 2 = 132 mg/L; a = $p < 0.05$; b = $p < 0.01$. (n=4)

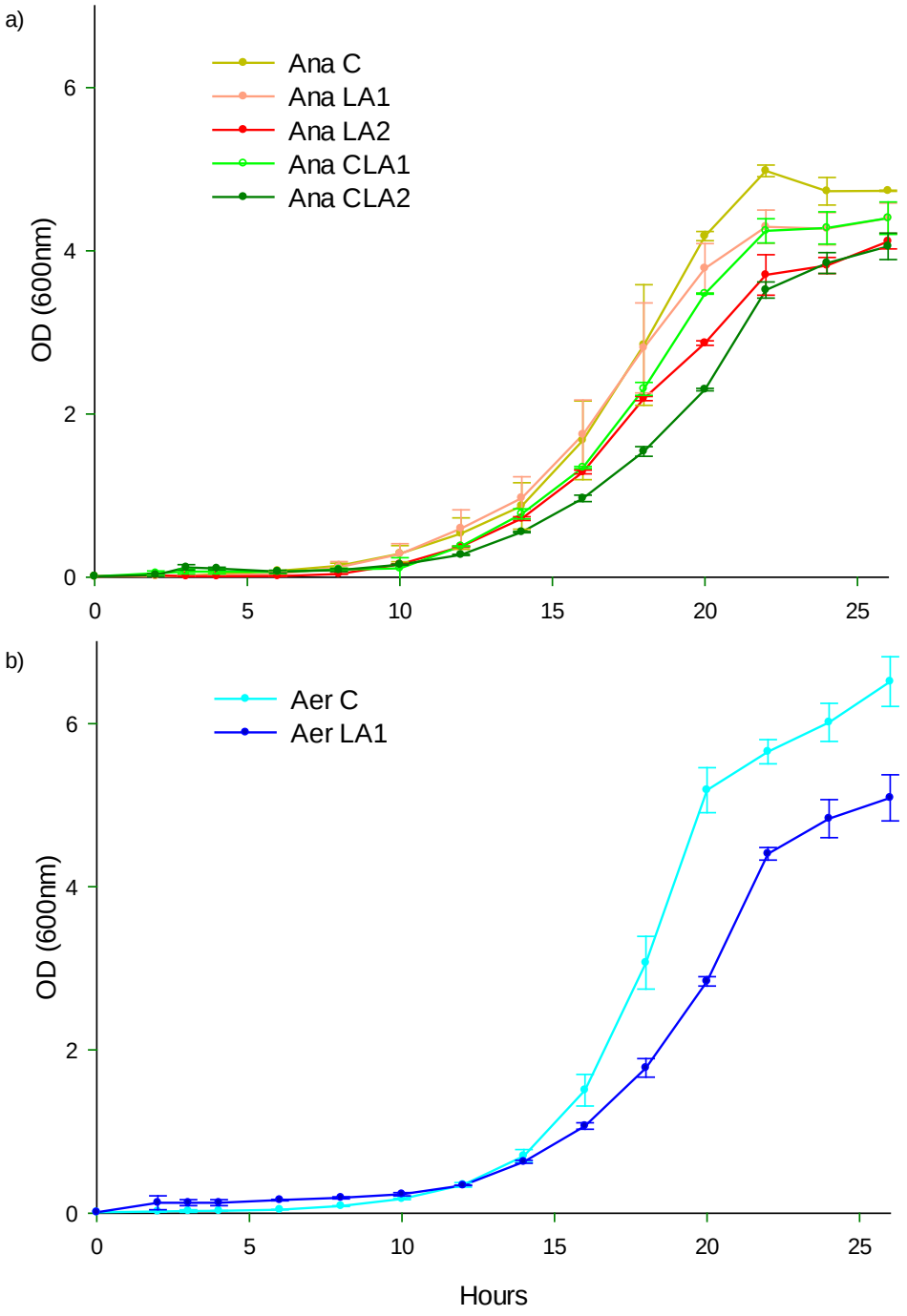

Figure 3.1. *Saccharomyces cerevisiae* growth on glucose supplemented and non-supplemented with different concentrations of linoleic and conjugated linoleic acids. The yeast cells were grown under anaerobic (Ana, a) and aerobic conditions (Aer, b). C = control; LA = linoleic acid supplementation, CLA = conjugated linoleic acid supplementation; 1 = 66 mg/L; 2 = 132 mg/L; n = 4.

3.3.2. The response of *S. cerevisiae* to linoleic and conjugated linoleic acids: extracellular metabolites

A total of 13 extracellular metabolites were identified in the spent culture medium samples (**Figure 3.2**). Surprisingly, *S. cerevisiae* responded to LA and CLA by increasing the glucose consumption rate ($p < 0.01$) and increasing ethanol production ($p < 0.05$) under anaerobic conditions (**Table 3.5**). This was not expected and since under anaerobic conditions, *S. cerevisiae* ferments glucose into ethanol to recycle NAD^+/NADH (alcoholic fermentation, Hahn-Hägerdal and Mattiasson (1982)), these results suggest that both LA and CLA supplementation increase the fermentation rate. This might be the way for the cell to produce more ATP required for linoleic acid transport (Hiltunen et al., 2003). On the other hand, *S. cerevisiae* performs alcoholic fermentation even under aerobic conditions if the levels of glucose are sufficiently high. This phenomenon is called the Crabtree effect (Crabtree, 1929). However, no significant difference in glucose and ethanol concentrations were found under aerobic conditions when comparing control cultures to the LA supplemented and non-supplemented flasks (**Table 3.5**). This indicates the concentration of glucose was low enough at the time of sampling to stop the Crabtree effect (**Table 3.5**).

Table 3.5. Glucose and ethanol concentration in spent media of *Saccharomyces cerevisiae* grown on glucose supplemented or not with different concentrations of linoleic acid and conjugated linoleic acid.

	Condition	Glucose g/L	Ethanol g/L
Anaerobic			
	C	2.0 ± 0.8	6.7 ± 0.5
	LA1	0.9 ± 0.8[a]	8.0 ± 1.0[a]
	LA2	0 ± 0[a]	10.2 ± 1.3[b]
	CLA1	1.2 ± 1.1[a]	9.3 ± 0.8[b]
	CLA2	1.1 ± 0.3[a]	10.2 ± 0.4[b]
Aerobic			
	C	3.2 ± 0.6	6.4 ± 0.2
	LA1	3.0 ± 1.4[c]	6.2 ± 0.6[c]

C = non-supplemented control; LA = linoleic acid supplementation, CLA = conjugated linoleic acid supplementation; 1 = 66 mg/L; 2= 132 mg/L; a = $p < 0.05$; b = $p < 0.01$; c = $p > 0.05$. (n=4)

Furthermore, LA and CLA seemed to affect the secretion and/or up-take of metabolites by *S. cerevisiae* (**Figure 3.2**). For instance, *S. cerevisiae* secreted higher levels of two amino acids (proline and glutamic acid) and two fatty acids (octanoic and stearic acid) when compared to control cultures ($p < 0.05$, **Figure 3.2**). In particular, glutamic acid and stearic acid in the spent culture media showed an increase in levels under all LA-supplemented growth conditions regardless of the presence or absence of oxygen. Moreover, as previously observed by Pinu (2013), *S. cerevisiae* produced more glycerol than the control cultures when LA was supplemented to the grape juice medium at concentration of 132 mg/L .

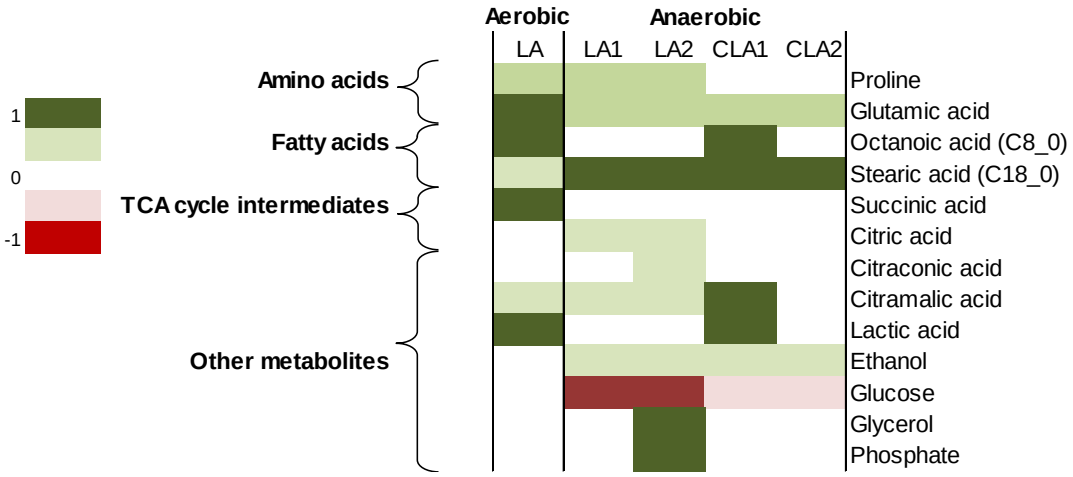

Figure 3.2. Fold changes in the extracellular metabolites levels of *Saccharomyces cerevisiae* grown on glucose in the presence or absence of linoleic acid isomers. Only the metabolites showing a significant change in comparison to the control ($p < 0.05$) are shown. The green and red colours indicate metabolites secreted or taken up, respectively. The white colour represents no significant change of the compound level ($p > 0.05$) when the linoleic acid isomer is supplemented compared to the control. LA = linoleic acid supplementation; CLA = conjugated linoleic acid supplementation; 1 = concentration of 66 mg/L; 2 = concentration of 132 mg/L.

3.3.3. The response of *S. cerevisiae* to linoleic and conjugated linoleic acids: intracellular metabolites

A total of 64 metabolites in the intracellular samples of *S. cerevisiae* were identified using our in house-MS library. Among them, there were 19 proteinogenic amino acids, 8

non-proteinogenic amino acids, 16 fatty acids, 6 TCA cycle intermediates and 14 other metabolites from central carbon metabolism (**Figure 3.3**).

Supplementation with LA and CLA affected the intracellular levels of central carbon metabolism intermediates. For example, the level of pyruvic acid decreased considerably (25-39%, $p < 0.01$) under anaerobic conditions in response to LA and CLA supplementation, while no significant change was observed in its level under aerobic conditions. Clearly, this response of *S. cerevisiae* to the linoleic acid isomers is in agreement with the increased production of ethanol from glucose when compared to the control cultures based on the extracellular metabolite analysis since pyruvic acid is the key precursor for ethanol.

Furthermore, under anaerobic conditions, LA and CLA supplementation significantly increased ($p < 0.05$, **Figure 3.3**) the level of TCA cycle intermediates and proteinogenic amino acids at both supplementation concentrations. However, supplementing with 132 mg/L resulted in the increase of a higher number of proteinogenic amino acids and TCA cycle intermediates compared to 66 mg/L. In particular, the intracellular accumulation of amino acids in response to linoleic acid isomers suggests that these cell building blocks were less incorporated into biomass compared to the control because their biosynthesis in *S. cerevisiae* is tightly controlled by feedback regulation (Chubukov et al., 2014), which is in agreement with the biomass concentration results (**Table 3.4**).

On the contrary, under aerobic conditions only three proteinogenic amino acids and TCA cycle intermediates showed significant increases in concentration when LA was supplemented but the maximum biomass titre was still lower than for the control cultures (**Table 3.4**). This result suggests that even if the growth rate and biomass production are negatively affected by the linoleic acid isomers under both anaerobic and aerobic conditions, the mechanisms regulating yeast growth are different based on the presence or absence of oxygen.

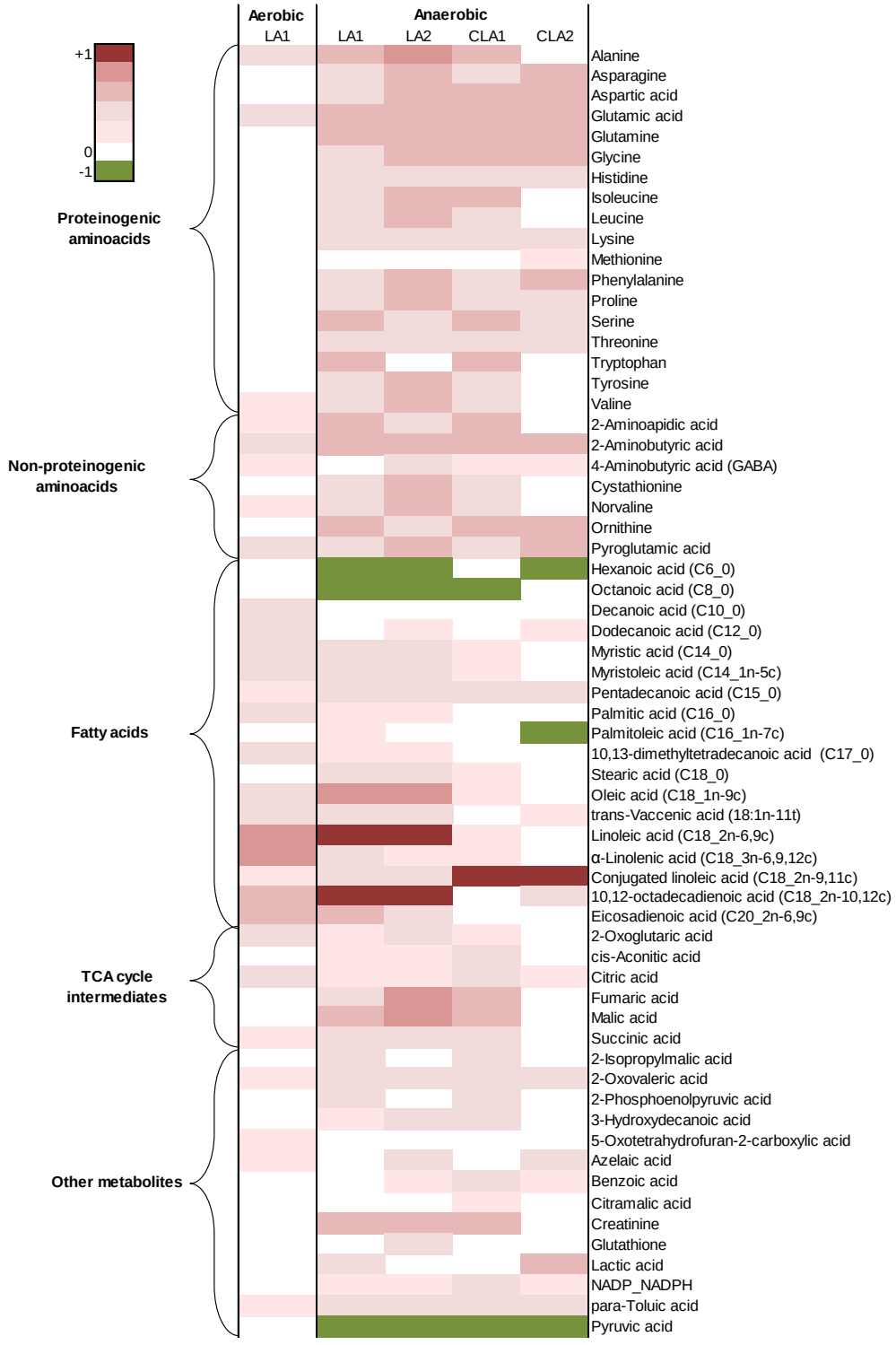

Figure 3.3. Fold changes of the intracellular metabolites in *Saccharomyces cerevisiae* in response to linoleic and conjugated linoleic acids. The red and the green colours represent an increase and a decrease in the metabolite levels, respectively, if compared to the control cultures ($p < 0.05$); the white colour represents a non-significant change in levels ($p > 0.05$). LA = linoleic acid supplementation; CLA = conjugated linoleic acid supplementation; 1 = concentration of 66 mg/L; 2 = concentration of 132 mg/L.

The analysis of intracellular fatty acids showed that over 97% of the supplemented LA and CLA were taken up by *S. cerevisiae* cells under both anaerobic and aerobic conditions (**Figure 3.3**), indicating their prompt transportation into the cells. The presence of these unsaturated fatty acids clearly affected the level of other intracellular fatty acids (**Figure 3.3**). Interestingly, the intracellular concentration of CLA increased 2 to 5 fold ($p < 0.001$) in response to LA supplementation and this has not been reported before. In addition, under anaerobic conditions, the intracellular levels of two medium chain fatty acids (MCFAs), hexanoic and octanoic acids decreased consistently ($p < 0.05$) when LA is supplemented at both concentrations, whereas this was not observed in CLA supplemented cultures (**Figure 3.3**). However, 15 fatty acid levels increased ($p < 0.05$) in response to the supplementation of LA and CLA under anaerobic conditions and 12 under aerobic conditions. Once again, the accumulation of these building blocks confirms and agrees with the reduction of biomass production (**Table 3.4**).

3.3.4. Pathway activity profiling (PAPi) analysis

Using the profile of intracellular metabolites and their relative concentrations, we carried out a pathway activity profiling analysis (PAPi, **Figure 3.4**) which is a hypothesis-generating tool developed in our laboratory (Aggio et al., 2010). Under both anaerobic and aerobic conditions, most of the metabolic pathways were up regulated in response to both LA and CLA supplementation.

Under anaerobic conditions, the pentose phosphate pathway seemed to have been down-regulated while glycolysis and pyruvate metabolism were upregulated in response to the higher dose of LA and CLA supplemented to the media. This result was supported by the lower concentration of glucose, ethanol and pyruvate at the time of sampling, suggesting that the supplementation with linoleic acid isomers increased the production of ethanol, most probably through an increase in the flux of glycolysis and fermentation pathways. Moreover, according to PAPi, the TCA cycle and the biosynthesis

of amino acids were up-regulated in response to linoleic and conjugated linoleic acids. This was in accordance with the higher fermentation rate observed in the metabolic profiles of LA and CLA supplemented cultures. Interestingly, this suggests that pyruvate, with its high turnover caused by LA and CLA supplementation, not only was used to produce more ethanol but also more TCA cycle intermediates and consequently, amino acids which accumulated rather than being used for biomass production. As expected, the pathway for fatty acid elongation also seemed to be up-regulated in response to LA, which agrees with the higher levels of fatty acids reported in the metabolic profile (**Figure 3.3**)

Contrary to the anaerobic conditions, under aerobic conditions glycolysis shows a lower activity in response to LA. This was expected because, in presence of oxygen and not under the Crabtree effect, *S. cerevisiae* can perform aerobic respiration which produces a higher energy yield than alcoholic fermentation (Lehninger et al., 2005). Therefore, if the majority of the ATP is produced during respiration, the cells do not need to upregulate glycolysis (as they do under anaerobic conditions) to overcome the energy needed to transport and activate LA (Hiltunen et al., 2003).

Moving on from central carbon metabolism, PAPi indicates that under both anaerobic and aerobic conditions glutathione metabolism was up-regulated when compared to the control. Interestingly, glutathione is an important antioxidant in biological systems and protects the cells from oxidative stress. Cell oxidative stress can be caused by PUFAs, linoleic acid included (Cury-Boaventura et al., 2004; Ferreira et al., 2011; Pompeia et al., 2003). In this case it was most probably a metabolic response to the oxidative stress related to the presence of the linoleic acid isomers and the higher ethanol concentration produced as a consequence.

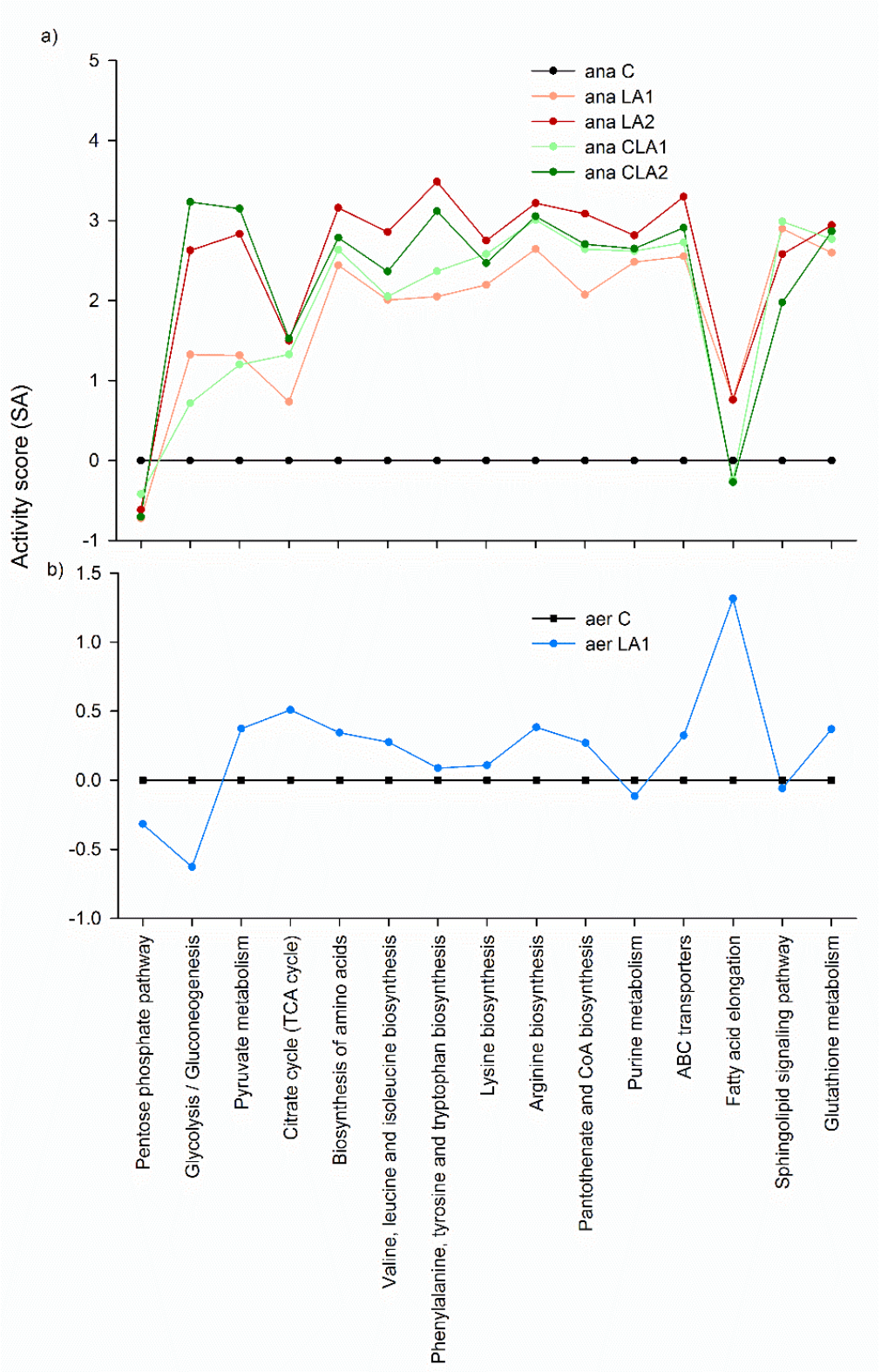

Figure 3.4. Metabolic pathway activity profile based on intracellular metabolite data of *Saccharomyces cerevisiae* growing on glucose under anaerobic (a) and aerobic (b) conditions in the presence or absence of linoleic acid isomers. C = control; LA = linoleic acid supplementation, CLA = conjugated linoleic acid supplementation; 1 = 66 mg/L; 2 = 132 mg/L; a = $p < 0.05$; b = $p < 0.01$; n=4

3.3.5. The fate of linoleic acid in *S. cerevisiae* anaerobic cultures

The fate of linoleic acid in *S. cerevisiae* cells was investigated by combining minimal mineral media cultured with ^{13}C-linoleic acid.

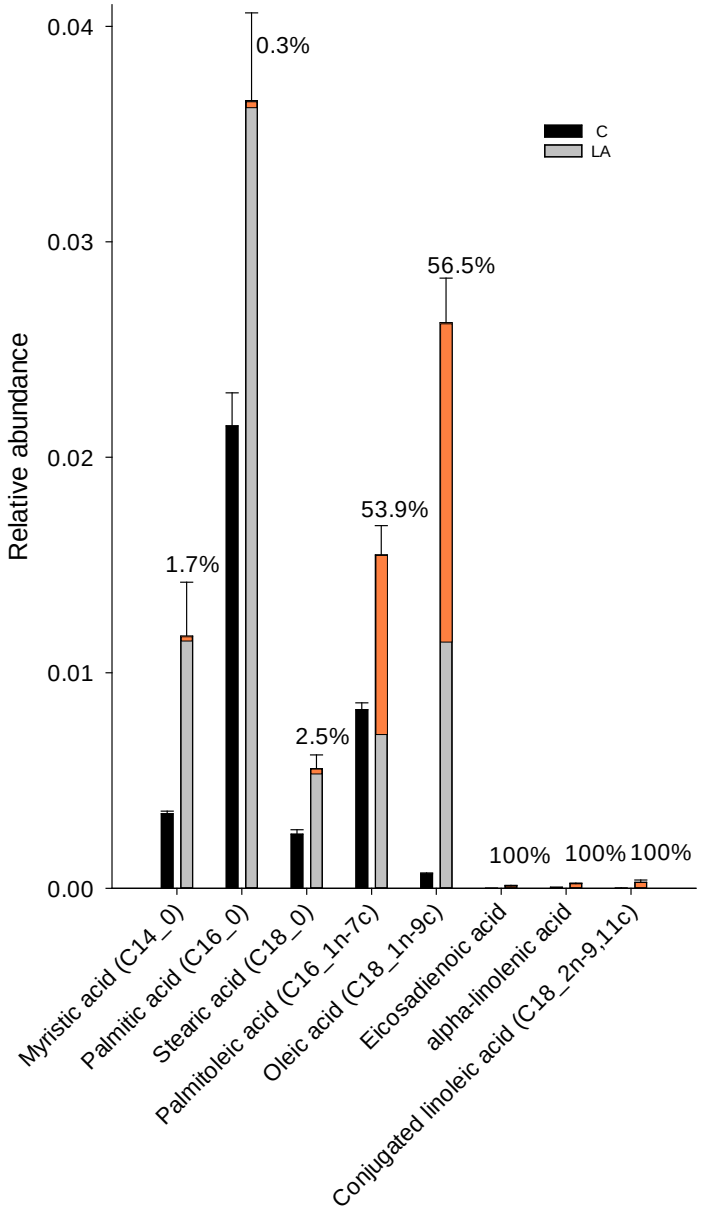

Figure 3.5. Free fatty acids found with increased level of ^{13}C in *Saccharomyces cerevisiae* cells grown on minimal mineral media with glucose supplemented with ^{13}C-linoleic acid under anaerobic conditions.. C = control (with no linoleic acid supplementation); LA = ^{13}C-linoleic acid supplementation at 66 mg/L. Orange colour represents the percentage of the molecules that were uniformly ^{13}C-labelled.

The intracellular metabolite profile of anaerobic and aerobic cultures was analysed and 8 isotopically labelled fatty acids were identified (**Figure 3.5**). The results of the anaerobic cultures indicate that *S. cerevisiae* transformed linoleic acid preferentially into two monosaturated fatty acids: oleic and palmitoleic acids, with 56.5 and 53.9% of the molecules uniformly labelled, respectively (**Figures 3.5**, **3.6**). At first the linoleic acid was most probably converted into oleic acid with the saturation of a double bond (Khor & Uzir, 2011). This reaction is well described for *S. cerevisiae* and is not oxygen-dependant. However, the subsequent conversion to the labelled palmitoleic acid was unexpected because it means that the labelled oleic acid lost two carbon units, as usually happens under aerobic conditions through β-oxidation, such a mechanism under anaerobic conditions has not been described yet.

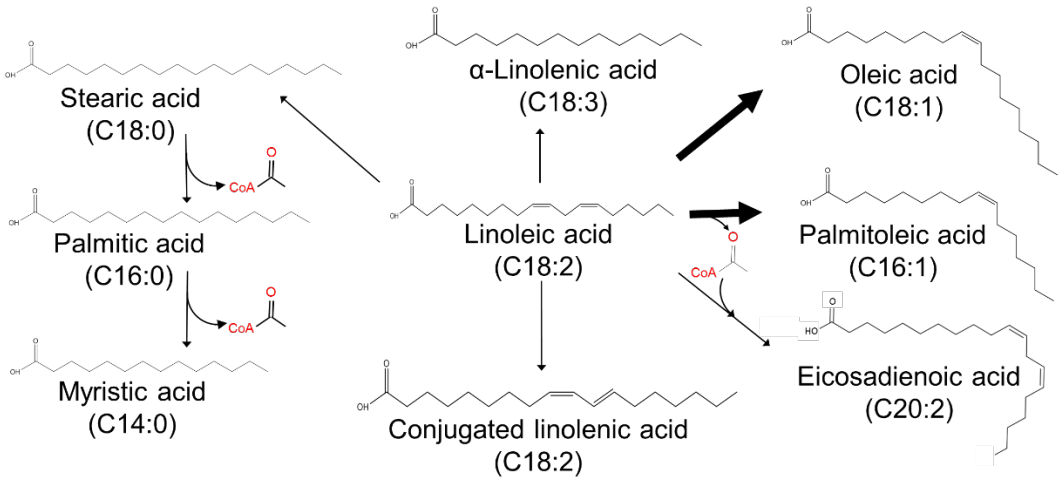

Figure 3.6. Metabolic fate of linoleic acid in *Saccharomyces cerevisiae* cells grown on glucose supplemented with ^{13}C-linoleic acid under anaerobic condition. The thicker arrows indicate the major products of linoleic acid metabolism.

Interestingly, three polyunsaturated fatty acids, α-linolenic acid, a conjugated linoleic acid isomer and eicosadienoic acid were found with their levels increased in response to linoleic acid supplementation albeit in low concentration. All molecules were uniformly ^{13}C-labelled, indicating that *S. cerevisiae* had synthesised them directed from

linoleic acid supplemented to the cultures (**Figure 3.6**). Eicosadienoic acid is known to be synthesised from linoleic acid through a pathway which does not require oxygen (Lehninger et al., 2005; Riezman, 2007). However, the formation of α-linolenic acid, a fatty acid with three double bonds, was not expected for two reasons: the first one is that *S. cerevisiae* has only one desaturase, OLE1 (Stukey et al., 1989), which is not capable of producing polyunsaturated fatty acids with more than two double bonds (Chemler et al., 2006; Yazawa et al., 2009). The second reason is that the desaturation reaction requires molecular oxygen. These results could suggest that some oxygen might have leaked into the flasks. However, we do not believe that is the case due to all the precautions taken, such as saturating the media and the head space with nitrogen gas before the inoculation, and sealing the rubber stops with glycerol and parafilm. On the contrary, this may indicate that *S. cerevisiae* has a novel mechanism for desaturation independent of oxygen as seen in other microorganisms (Feng & Cronan, 2011). Notably, the supplementation of isotopically labelled linoleic acid to the medium induced *S. cerevisiae* to produce labelled conjugated linoleic acid, which clearly showed that the cells are able to synthesise a conjugated form using linoleic acid as substrate. Even if the isomerase responsible for the conversion (Kepler & Tove, 1967) has not yet been described for *S. cerevisiae,* all the conjugated linoleic acid detected was synthesised from the linoleic acid provided. This suggests the presence of an enzyme able to carry out this reaction.

A small amount (less than 3%) of three saturated fatty acids was labelled, suggesting that a minor fraction of linoleic acid had two double bonds reduced (producing stearic acid) and the carbon chain shortened (producing palmitic and myristic acid) (**Figure 3.6**). This is another remarkable finding because under anaerobic conditions *S. cerevisiae* conversion of linoleic acid to shorter chain fatty acids would require β-oxidation. However, the strict anaerobic conditions that *S. cerevisiae* was grown under should have prevented β-oxidation from occurring.

Differently from the anaerobic cultures, no labelled compounds were found in the aerobic cultures supplemented with ^{13}C-labelled linoleic acid. Under aerobic conditions, we

expect that linoleic acid would be easily broken down through β-oxidation into glycerol and acetyl-CoA molecules to produce energy. Since acetyl-CoA and glycerol are involved in many pathways, if they were coming from the labelled LA, we should have detected some labelled compounds originating from them. However, the concentration of linoleic acid was small compared to the main carbon source, glucose, which was not labelled. Consequently, the proportion of acetyl-CoA and glycerol labelled with ^{13}C was very low compared to the ^{12}C coming from the glucose, hence the reason why no labelled compounds were detected under aerobic conditions.

3.4. Discussion

In this study, the effect of the linoleic acid and one of its conjugated forms on *S. cerevisiae* metabolism was investigated using metabolomics platforms. This is the first study that has investigated the influence of linoleic acid on the metabolic pathways of *S. cerevisiae*. The results demonstrated that linoleic acid and conjugated linoleic acid clearly affected *S. cerevisiae* metabolism inducing changes in both the intracellular and extracellular metabolite profiles.

The main consequence of the linoleic acid supplementation was found under anaerobic conditions. As supported by the metabolomics analysis, *S. cerevisiae* increased its fermentative activity in response to linoleic acid and its isomers (**Figures 3.2, 3.3**). In fact, *S. cerevisiae* produced ethanol from pyruvate at a faster rate (**Table 3.5** and **Figure 3.3**) clearly indicating an up-regulation of the fermentation. A higher fermentation rate implies that the cells need more energy. Under this condition, in fact, the only way for the cells to produce ATP is through glycolysis; therefore the alcoholic fermentation must be up-regulated in order to regenerate the NAD^+ required for the glycolytic pathway (Ishtar Snoek & Yde Steensma, 2007). The increase in energy demand when linoleic acid is available is due to its transportation into the cell. Interestingly, *S. cerevisiae* uptakes and activates linoleic acid using two ATP-requiring acyl-CoA synthetases, FAA1 and FAA4 (Færgeman et al., 2001). When this fatty acid is available under anaerobic conditions it is promptly taken up with an energy expense because the oxygen-dependent enzymes necessary for its synthesis are inactive (Andreasen & Stier, 1953; Erwin, 2012). Moreover, it is known that once in the cytoplasm, the cells compartmentalise linoleic acid into the peroxisome (Veenhuis et al., 1987), most probably due to its toxicity (Ferreira et al., 2011) and again using ATP (Hettema et al., 1996; Swartzman et al., 1996), which increase the energy demand. In this environment the energy is mainly focused onto linoleic acid transport and consequently less available for the other cell pathways. In fact, as the amino acid and fatty acid accumulation confirms (**Figure 3.3**), the biosynthesis of building blocks was reduced

in the presence of linoleic acid because it is an ATP-dependant process. As a consequence, the cell growth rate and biomass titre were negatively affected by linoleic acid supplementation (**Table 3.4**). Under aerobic conditions the linoleic acid is still transported into the cell increasing the energy demand but no increase of the fermentation rate was observed (**Table 3.5** and **Figure 3.3**). This was expected because when *S. cerevisiae* is not under the Crabtree effect (as glucose was not sufficiently high, **Table 3.1**), it can use the respiratory electron chain which is much more efficient in producing ATP than alcoholic fermentation. However, more energy is spent on linoleic acid transport rather than for biomass production (**Table 3.4**).

S. cerevisiae was also grown under anaerobic conditions supplementing the medium with isotopically-labelled ^{13}C-linoleic acid to follow its fate into the cell. It is clear that linoleic acid was transported into the cell from the external medium. Part of that pool remained as free fatty acid while another part was transformed in other fatty acids. The conversion of linoleic acid involved changes in the length of the carbon chain and in the number of double bonds (**Figure 3.6**). Surprisingly, under anaerobic conditions linoleic acid was converted into palmitoleic, palmitic and myristic acid. However, this was not expected under such conditions since all this conversions usually involves β-oxidative steps requiring oxygen. Current literature does not indicate that yeasts accomplish β-oxidation without oxygen. However, it is not unusual for bacteria (Campbell et al., 2003; Jackson et al., 1999; McInerney et al., 1981) In fact, alternative anaerobic pathways to β-oxidation were described for an E. *coli* strain (Campbell et al., 2003), for *Syntrophus aciditrophicus* (Jackson et al., 1999) and for *Syntrophomonas wolfei* (McInerney et al., 1981). This discovery might represent the starting point for a deeper analysis of the pathways involved in a potential *S. cerevisiae* “anaerobic β-oxidation” pathway.

3.5. Conclusions

Based on the results of the comprehensive intracellular and extracellular metabolomics analysis, it can be concluded that linoleic acid availability in the environment significantly affects *S. cerevisiae* metabolism. The increase in the synthesis of ethanol could be particularly interesting to the biofuel industry which utilises ethanol broadly to convert chemical energy into mechanical energy. At present, 90 billion litres of bio-ethanol is produced every year all over the world (Badwal et al., 2015). Ethanol production can be generated through the microbial fermentation of food crops like corn (US) and sugarcane (Brazil) or inedible parts of plants. This study shows that a potential more efficient way to produce ethanol is the supplementation of linoleic and conjugated linoleic acid to *S. cerevisiae* cultures. Moreover, according to my results, linoleic acid itself seemed to be more promising when compared to its conjugated form because even through a similar amount of ethanol was produced, linoleic acid is much cheaper. This novel information can be used by the bio-ethanol industry to study the possible supplementation with these fatty acids to the substrate of choice. Moreover, this study indicates that *S. cerevisiae* might be able to perform an alternative anaerobic pathway to β-oxidation that has not yet been described in the literature.

CHAPTER FOUR

The effect of linoleic acid on the lipidome of *Saccharomyces cerevisiae*

Francesca Casu[1], Sergey Tumanov[2], Farhana R. Pinu[3], Eliezer Stefanello[4], David R. Greenwood[1,3] and Silas G. Villas-Bôas[1]

[1] School of Biological Sciences, the University of Auckland, 3A Symonds Street, Auckland 1010, New Zealand,

[2] Cancer Research UK Beatson Institute, Garscube Estate, Switchback Road, Glasgow G61 1BD, UK and

Institute of Cancer Sciences, University of Glasgow, Garscube Estate, Switchback Road, Glasgow G61 1BD, UK.

[3] The New Zealand Institute for Plant and Food Research Limited, 120 Mt Albert Rd, Sandringham, Auckland 1025, New Zealand

[4] Department of Biochemistry, Institute of Chemistry, University of São Paulo, Av. Prof. Lineu Prestes, 748, São Paulo, SP, Brazil

The content of this chapter has not been published

4.1. Introduction

The presence of two isomers of linoleic acid in the environment influences fermentation parameters and cell metabolism of aerobic and anaerobic cultures of *Saccharomyces cerevisiae* (**Chapter 3**). Growth rate and biomass decreased in response to linoleic acid supplementation, and fermentation rate increased under anaerobic conditions. In tandem, the abundance of many metabolites was altered and, among them, all the fatty acids identified increased in response to supplementation with linoleic acid isomers (**Figure 3.3**).

Fatty acids have a key role in the lipid metabolism representing an important component of the lipids themselves (Klug & Daum, 2014). Therefore, it is not surprising that these molecules influence the cell lipidome. In fact, previous studies have already correlated the supplementation of fatty acids or fatty acid-containing molecules to changes in *S. cerevisiae* lipids (Beltran et al., 2008; Jollow et al., 1968). For example, an increase in levels of phospholipids, ergosterol and unsaturated fatty acids was shown in response to supplementation with ergosterol and Tween80 under both aerobic and anaerobic cultures (Jollow et al., 1968). Under anaerobic growth in particular, a prompt uptake of external sources of linoleic acids by yeast cells was observed (Chen, 1980, Chapter 3) because *S. cerevisiae* cannot synthesise unsaturated fatty acids in the absence of oxygen (Hiltunen et al., 2003). However, no comprehensive *S. cerevisiae* lipidomics study in response to linoleic acid has been reported to date, even though it greatly influences yeast cell metabolism (**Chapter 3**). For this reason and due to the key role of the fatty acids in lipid metabolism, this current chapter presents a shotgun lipidomic study of *S. cerevisiae* in response to linoleic acid supplementation under both aerobic and anaerobic growth conditions.

4.2. Material and methods

4.2.1. Chemicals

All chemicals used in this study were of analytical grade. Chloroform and methanol and ammomium bicarbonate were obtained from Merck (Darmstadt, Germany). Δ9-cis,Δ12-cis-linoleic acid, Δ9-cis, Δ11-trans-linoleic acid were purchased from Nu Chek Prep. Inc (Elysian, USA). Glucose and nonadecanoic acid were obtained from Sigma-Aldrich (St. Louis, MO). The internal standard for lipidome analysis triethanolamine trimyristate (TEM) was purchased from Omics Biochemicals Limited (Auckland, New Zealand). Grade 1 water 77 (BARNSTEAD® NANOpure DIamond™ Water Purification System, Waltham, MA) was used to prepare most of the solutions.

4.2.2. Yeast strain

Saccharomyces cerevisiae EC1118 (Lallemand, Montreal, Canada) was the yeast strain used for all fermentations and was maintained on YPD agar medium (glucose 20 g/L, peptone 20g/L, yeast extract 10 g/L, agar 20 g/L) at 30°C.

4.2.3. Flask culture

S. cerevisiae EC1118 was cultivated according to the protocol described in **Chapter 3.2.3**. Briefly, the cells were grown in shaking flasks aerobically and anaerobically at 30°C in a minimal mineral medium (20 g/L of glucose, salts, vitamins and trace metals (Verduyn et al., 1992)) that was supplemented with one of two isomers of linoleic acid, Δ9-*cis*,Δ11-*cis*-linoleic acid (LA) or Δ9-*cis*,Δ12-*trans*-conjugated linoleic acid (CLA), at two different concentrations (**Table 4.1**). When the cells reached 2.5 OD_{600}, the maximum OD for all the culture to be in exponential phase, 1 mL of culture from each flask was sampled and stored at -80°C.

Table 4.1. The concentration of linoleic acid and its isomers in minimal mineral media used to culture *Saccharomyces cerevisiae* EC1118 under different experimental conditions.

Condition	Isomer	Concentration (mg/L)
Anaerobic		
Ana C	-	-
Ana LA1	9-cis,12-cis-Linoleic acid	66
Ana LA2	9-cis,12-cis-Linoleic acid	132
Ana CLA1	9-cis,11-trans-Conjugated linoleic acid	66
Ana CLA2	9-cis,11-trans-Conjugated linoleic acid	132
Aerobic		
Aer C	-	-
Aer LA1	9-cis,12cis-Linoleic acid	66

4.2.4. Cold-lipid extraction

The extraction of lipids was performed using a modified protocol described by Ejsing et al. (2009). All the solutions and samples were kept at 4°C prior and during the extraction. The cells were resuspended in 150 mM ammonium bicarbonate (pH 8) and ruptured with zirconia beads (0.5 mm). The cell lysates were diluted to 0.2 OD_{600} and 200 µL were transferred in amber GC-MS vials. An amount of 20 µL for each internal standard (5 mM chloroform solution of TEM for positive ionisation mode and 0.1 M chloroform solution of nonadecanoic acid for negative ionisation mode) were added. The extraction was accomplished by adding 990 µL of chloroform/methanol (17:1 $_{V/V}$) and after 120 min the lower organic phase was collected and the upper phase was re-extracted by adding 990 µL of chloroform/methanol (2:1 $_{V/V}$). The lower organic phase of the extract was collected and pooled with the previous one. The lipid extracts were concentrated under nitrogen gas and then they were dissolved in 100 µL of chloroform/methanol (1:2 $_{V/V}$).

4.2.5. *S. cerevisiae* shotgun lipidomics and data analysis

The shotgun lipidome analysis of *S. cerevisiae* cells was performed by direct infusion of the extracted lipid sample into a Q-Exactive™ orbitrap mass spectrometer

(Thermo Scientific™, San Jose, CA) equipped with an electrospray (ESI) ion source as described in Tumanov et al. (2015). Briefly, the samples were injected into the ESI source with a syringe pump at the flow rate of 5 µL/min. The positive and negative modes were used to analyse the different classes of lipids. For the top 100 most abundant ion peaks, tandem MS was performed. The settings of the source were as follow: sheath gas flow rate 6 (arbitrary units), auxiliary gas flow rate 5 (arbitrary units), sweep gas flow 4 (arbitrary units), spray voltage 3.1 kV for positive ion ESI and 4.0 kV for negative ion ESI, capillary temperature 150°C. The resolution of the mass spectrometer was 140000 and automatic gain control was 3 x 10^6 with a maximum injection time of 200 ms. Positive mode scan range was *m/z* 100-1500 and the negative mode was *m/z* 100-1000. Recorded data files (".raw" files) were extracted and lipids were identified by LipidSearch™ 4.1 software (Thermo Scientific). Data on all detected peaks were exported as an Excel datasheet. Relative concentrations of identified lipids in positive and negative modes were obtained via normalization to the intensity of TEM and nonadecanoic acid, respectively. The resulting spreadsheet was uploaded to MetaboAnalyst 3.0 (Xia et al., 2015) to perform Principal Component Analysis (PCA). A students' t-test was applied to determine whether the relative abundance of each identified lipid was significantly different between conditions. Lipids nomenclature was in accordance with Fahy et al. (2009).

4.3. Results

The lipidome of *S. cerevisiae* is known to be very diverse, containing a few hundred lipid species (Ejsing et al., 2009; Yetukuri et al., 2008). Our analyses identified and measured the relative abundances of 107 plasma-membrane lipid species that constituted 15 lipid classes belonging to three categories, i.e. glycerolipids, glycerophospholipids and sphingolipids (**Figure 4.1**).

The lipidome of *S. cerevisiae* control (non-supplemented) cultures showed a lower abundance of glycerolipids and sphingolipids when grown under aerobic conditions compared to anaerobic growth. In particular the level of triglycerides (TG), which are storage lipids, decreased most probably because preference was given to biomass formation under aerobiosis (**Table 3.4**), and consequently lipid metabolism favoured the biosynthesis of phospholipids rather than triglyceride production. In contrast, the glycerophospholipids showed a more variable pattern. The data showed an increase in the amount of glycerophosphates (PA) and a decrease of lyso-glycerophosphates (LPA) under anaerobic conditions whereas the opposite trend was observed under aerobic conditions. Therefore, PA biosynthesis may be more active in the absence of oxygen since LPA is the direct precursor of PA (Klug & Daum, 2014) .

Linoleic and conjugated linoleic acid supplementation of the *S. cerevisiae* ferments also had an effect on the abundances of lipid classes. Under aerobic conditions, the relative abundance of all the lipid classes in *S. cerevisiae* cultures supplemented with linoleic acid was reduced compared to the non-supplemented cultures. Interestingly, this might suggest that linoleic acid is responsible for a possible reduction in total lipid content in the cell. Even under anaerobic conditions, there was a general decrease in the abundance of glycerolipids and sphingolipids in response to linoleic and conjugated linoleic acid at both supplemented concentrations (**Figure 4.**). However, the relative concentrations of lyso-phospholipids (lyso-glycerophosphates, LPA; lyso-glycerophosphoglycerols, LPG; and lyso-phosphoinositols, LPI) increased while the relative phospholipid concentration

decreased (**Figure 4.1**). The lyso-forms of the glycerophospholipids are their direct precursors. Therefore this increase in lyso-phospholipids suggests a reduction of glycerophospholipid biosynthesis. Interestingly, this is further evidence for linoleic acid reducing overall lipid biosynthesis in *S. cerevisiae* cells.

The effect of linoleic and conjugated linoleic acid on the *S. cerevisiae* lipidome was determined by principal component analysis (PCA, **Figure 4.2**) using 107 lipid species. A clear separation was visible when linoleic and its conjugated form were supplemented to the media under both anaerobic and aerobic conditions regardless of the concentration supplemented. This suggests that even a small difference in linoleic or conjugated linoleic acid concentration (from 66 to 132 mg/L) can markedly affect the whole yeast lipidome.

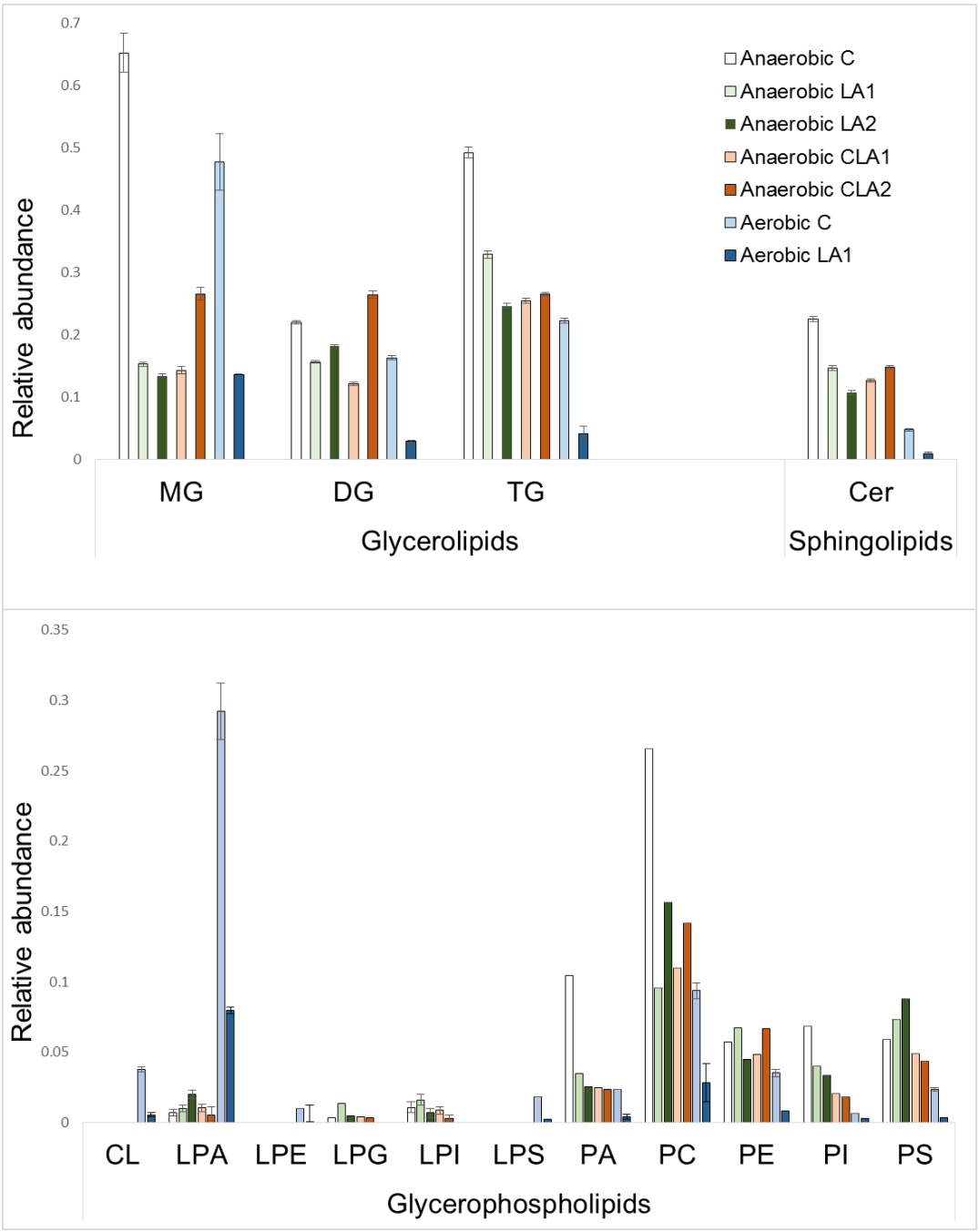

Figure 4.1. Relative abundance of different lipid classes in *Saccharomyces cerevisiae* in response to linoleic acid. C = control; LA = linoleic acid supplementation; CLA = conjugated linoleic acid supplementation; 1 = concentration of 66 mg/L; 2 = concentration of 132 mg/L; MG = Monoglycerides; DG = Diglycerides; TG = Triglycerides; Cer = Ceramides; CL = Cardiolipins; PA = Glycerophosphates (LPA for lyso species); PC = Glycerophosphocholines; PE = Glycerophosphoethanolamines (LPE for lyso species); PI = Glycerophosphoinositols (LPI for lyso species); PS = Glycerophosphoserines (LPS for lyso species); LPG = Lyso - glycerophosphoglycerols.

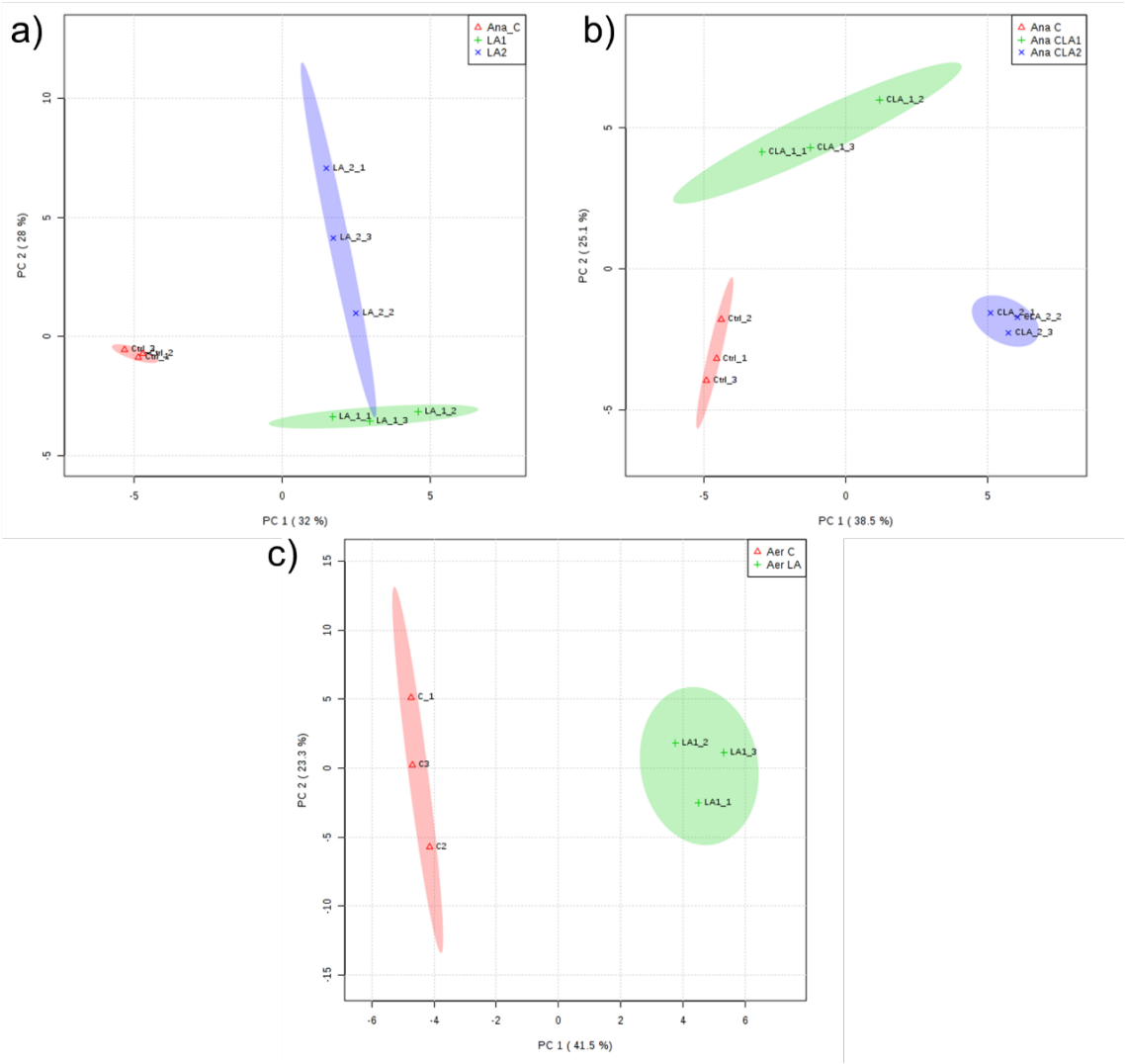

Figure 4.2. Two-dimensional projections of principal component analysis (PCA) using 107 statistically significant identified lipids. *Saccharomyces cerevisiae* cultures were grown under anaerobic (a) and (b), and aerobic (c) conditions. C = control; LA = linoleic acid supplementation; CLA = conjugated linoleic acid supplementation; 1 = concentration of 66 mg/L; 2 = concentration of 132 mg/L.

Two triacylglycerols were among the lipids most affected by linoleic acid under anaerobic growth, showing a decrease in abundance (**Figure 4.3**). This was not surprising because the biosynthesis of triacylglycerols represents the storage of metabolic energy and since the linoleic acid transport into *S. cerevisiae* requires ATP, the cell cannot afford to put energy in storage. A similar trend of reduction in lipid abundance was observed in response to CLA supplemented at the highest concentration, except for a monoglyceride

(**Figure 4.4**). Even if the total monoglycerides produced by *S. cerevisiae* are less abundant in the presence of CLA than in its absence (**Figure 4.1**), this particular monoglyceride (16:0) deserves attention because it is derived from palmitic acid, a key intermediate of lipid metabolism. In fact, palmitic acid can either be used in the biosynthesis of ceramides (Obeid et al., 2002) or it can enter the glycerol phosphate pathway to become a monoglyceride (Reddy et al., 2008). CLA supplementation of *S. cerevisiae* anaerobic cultures appears to direct palmitic acid into its monoglycerides rather than into ceramides. In the presence of oxygen, the cells responded to linoleic acid supplementation by decreasing the levels of 12 lipids, which also points towards an overall reduction in lipid biosynthesis in response to linoleic acid (**Figure 4.5**).

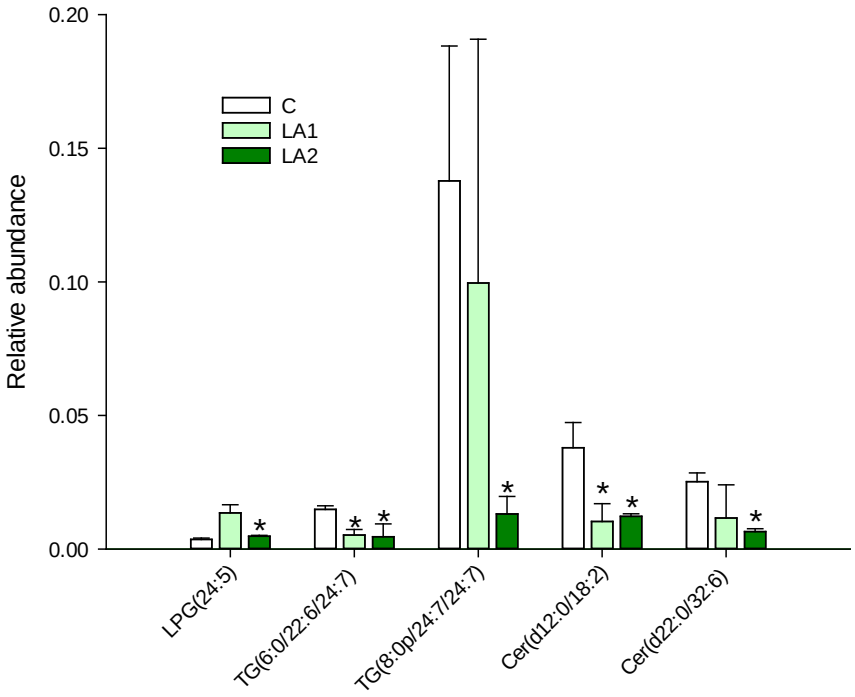

Figure 4.3. Relative abundance of *Saccharomyces cerevisiae* lipids in response to linoleic acid under anaerobic conditions. C = control; LA = linoleic acid supplementation; 1 = concentration of 66 mg/L; 2 = concentration of 132 mg/L. * = $p < 0.05$. n=3.

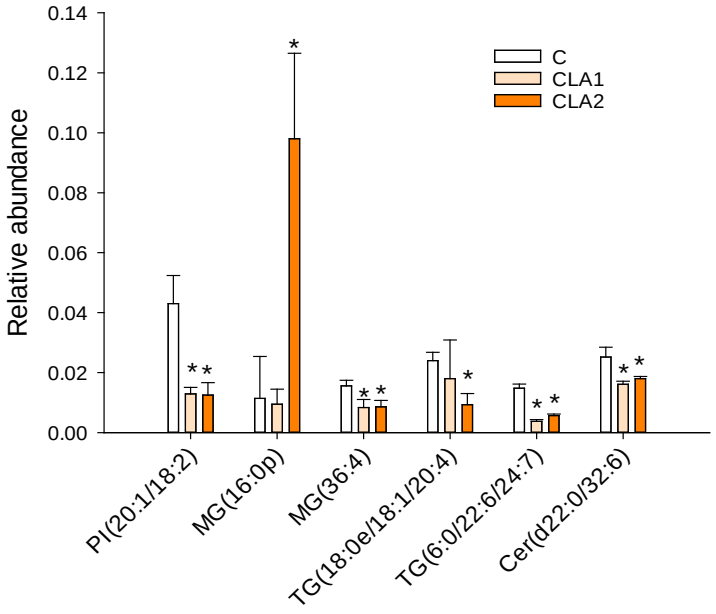

Figure 4.4. . Relative abundance of *Saccharomyces cerevisiae* lipids in response to conjugated linoleic acid under anaerobic conditions. C = control; CLA = conjugated linoleic acid supplementation; 1 = concentration of 66 mg/L; 2 = concentration of 132 mg/L. * = p<0.05. n=3.

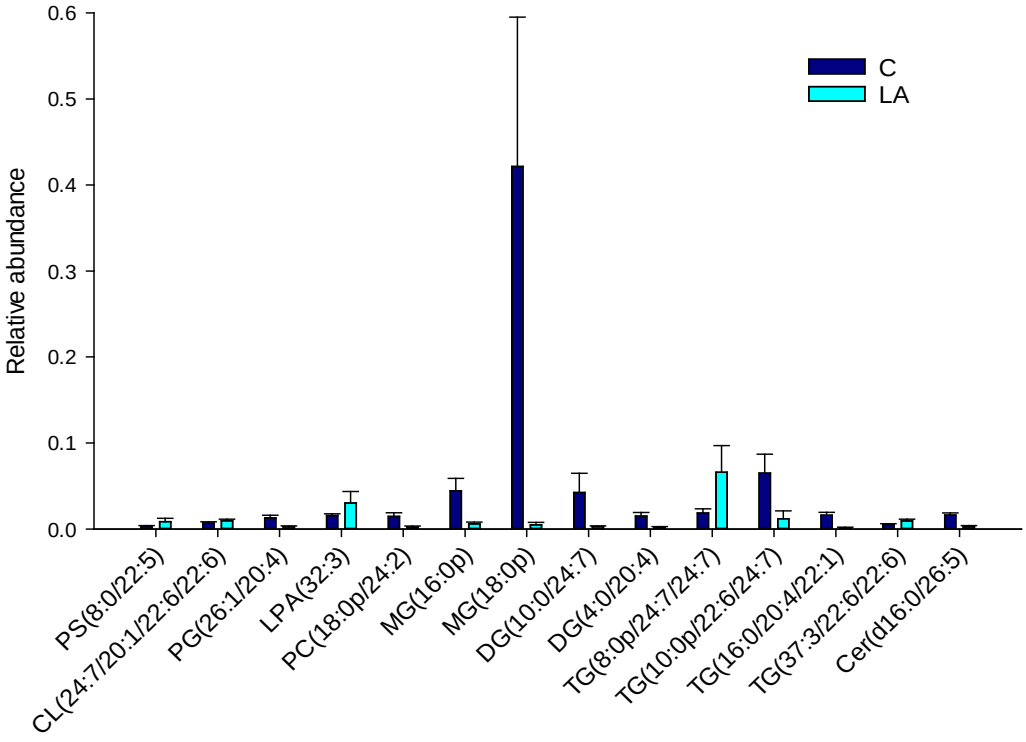

Figure 4.5. Relative abundance of *Saccharomyces cerevisiae* lipids in response to linoleic acid under aerobic conditions. C = control; LA = linoleic acid supplementation at 66 mg/L. p < 0.05 for each lipid. n=3

4.4. Discussion

This study investigated the effect of linoleic and conjugated linoleic acids on the *S. cerevisiae* lipidome using a shotgun lipidomic strategy. The results showed that the supplementation of the two linoleic acid isomers to *S. cerevisiae* cultures induced significant changes in the yeast lipid profile both aerobically and anaerobically.

The most evident *S. cerevisiae* response to linoleic acid supplementation was a clear reduction in total lipid content of the cells, which was independent from oxygen availability (**Figure 4.1**). This suggests that the supplementation with linoleic acid may down-regulate lipid biosynthesis in yeast cells. It has been shown previously that *S. cerevisiae* lipid content can decrease under anaerobic conditions because of ethanol production (Da Silveira et al., 2003). Data showed that linoleic acid absorption by the cells increased ethanol production under anaerobic conditions (**Chapter 3**), which also leads to a decrease of the lipid content as observed. However, this does not explain the reason why the amount of lipids dropped under aerobic conditions as well. An explanation may be found analysing the lipid biosynthetic pathways. Glycerolipids and glycerophospholipids are synthesised from a common precursor, phosphatidic acid (PA) which also acts as signalling molecule in the transcription of glycerophospholipid biosynthesis (Carman & Henry, 2007). PA biosynthesis is ATP-dependant and occurs through two sequential acylation reactions of glycerol-3-phosphate (Lehninger et al., 2005). Even the ceramides need ATP to be synthesised by a ceramide synthase from a sphingosine precursor (Dickson & Lester, 2002). This means that all the lipid classes identified in *S. cerevisiae* in this experiment are dependent on ATP for their biosynthesis (**Figure 4.6**).The transport of linoleic acid into *S. cerevisiae* cell is energy-dependent (Hettema et al., 1996; Hiltunen et al., 2003). Consequently, this may reduce the ATP pool in the cell when *S. cerevisiae* is growing in the presence of this unsaturated fatty acid, decreasing the energy available for other energy-dependent metabolic processes such as lipid biosynthesis (**Figure 4.6**) as well as cell division and biomass production as demonstrated in **Chapter 3**.

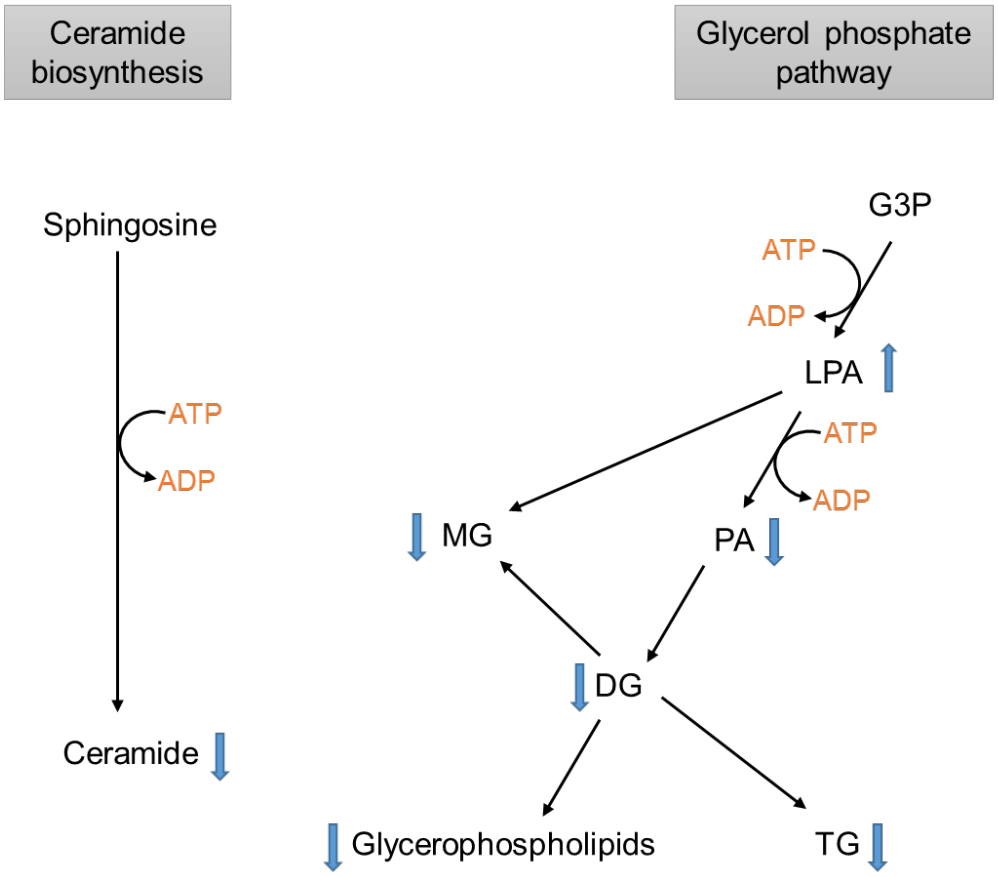

Figure 4.6. Energy requirements during lipid biosynthesis in *Saccharomyces cerevisiae*. The blue arrows indicate a decrease or an increase in lipid abundance in response to linoleic acid.

4.5. Conclusions

Based on the analysis of the *S. cerevisiae* lipidome, it can be concluded that linoleic acid and its isomers contribute to a decrease in the biosynthesis of cell lipids. The reason seems to be strongly correlated to the cell requirement for energy to take up linoleic acid. This information agrees with the *S. cerevisiae* metabolomics data that support the notion that yeast cells have less ATP available in the presence of linoleic acid. Due to the recent and strong interest around the microbial production of fatty acids (Steen et al., 2010), this information can be introduced potentially into the genome-scale model of *S. cerevisiae* to search for improvements in linoleic acid production.

CHAPTER FIVE

General discussion and future remarks

5.1. Overall discussion

The main aim of my thesis was to investigate the effect of linoleic acid isomers on *Saccharomyces cerevisiae* metabolism primarily in wine making but also in a laboratory-controlled environment. In order to do that, I have used metabolomics and lipidomics platforms because they provide a wide overview on the effect that linoleic acid might have on the wine produced by different *S. cerevisiae* strains and on the yeast metabolic pathways affected.

The first experiment was focused on the changes in the *S. cerevisiae* fermented Sauvignon blanc profile in response to an increase in concentration of linoleic acid in the grape juice. Fermenting with three different *S. cerevisiae* strains, I observed a general decrease in the acetylation process and a change to the wine profile affected by linoleic acid dependent on the strain used. This means that the different strains responded differently to the increased concentration of linoleic acid. Even though this information indirectly confirmed that linoleic acid affects *S. cerevisiae* metabolism through the changes in the wine profiles, no information could be clearly inferred from this data regarding the metabolic pathways involved. Moreover, wine is a very complex medium and contains hundreds of metabolites that could cover or disguise some important effects. For this reason, I moved to a laboratory-controlled environment, in which the cells were grown under aerobic and anaerobic conditions on glucose in a minimal mineral medium to reduce the number of variables. In particular, while in the wine making experiment I focused on changes in the wine properties and aroma compounds, now I analysed the intracellular and extracellular metabolites produced by *S. cerevisiae*. This gave me an idea of the pathways involved in the response to linoleic acid. In addition, since it is known that the fatty acids available in the environment affect cell lipids, it was appropriate and more exhaustive to investigate the effect of this particular fatty acid on the yeast lipidome.

5.2. What is the effect of linoleic acid on *S. cerevisiae* metabolism?

S. cerevisiae transports linoleic acid into the peroxisome to produce energy via β-oxidation when oxygen is available (Hettema et al., 1996; Hiltunen et al., 2003). There are many studies on the β-oxidative pathway and the aerobic fate of linoleic acid. However, less information is available under anaerobic conditions. In fact, under the lack of oxygen, fatty acid availability and acquisition from the environment are important requirements for the growth of *S. cerevisiae* (Ferreira et al., 2011). In particular, the cells rely on the external source of polyunsaturated fatty acids because they are unable to synthesise them under anaerobic conditions (Hiltunen et al., 2003). Among the polyunsaturated fatty acids, linoleic acid in particular was found to influence the aroma of the wine fermented by one strain of *S. cerevisiae* (Pinu et al., 2014a). I confirmed that, also observing that other *S. cerevisiae* strains are affected by linoleic acid influencing the production of wine aroma compounds, amino acids and antioxidant molecules (**Chapter 2**). However, when linoleic acid is taken up under anaerobic conditions and in the presence of a pro-oxidant like ethanol (Moradas-Ferreira & Costa, 2013), it can easily become a peroxide (Paltauf et al., 1992; Wagner et al., 1994). This is a strong reason for *S. cerevisiae* not to incorporate linoleic acid into its lipids, as observed in **Chapter 4**, but instead to compartmentalise it into the peroxisome. However, this transport into the peroxisome, just as the transport from the environment to the cytosol, requires ATP (Færgeman et al., 2001; Hettema et al., 1996). At this point, the cell needs energy to neutralise linoleic acid, via compartmentalisation, to overcome the stress. Interestingly, under anaerobic condition (and under the Crabtree effect in general) the main way to produce ATP is through substrate-level phosphorylation via glycolysis (**Figure 5.1**). However, the glycolytic pathway requires NAD^+, which is recycled through alcoholic fermentation (Ishtar Snoek & Yde Steensma, 2007). Consequently, there is an increase in ethanol production and of the fermentation rate to keep up with the ATP production by recycling NADH (**Chapter 3**). Since the cells are most probably using the

ATP to cope with the stress caused by linoleic acid and compartmentalising it into the peroxisome, they have less energy to use for the production of building blocks, such as proteins and lipids. Not surprisingly, this chain of events has a negative effect on biomass production and growth rate because the cells are using their energy to survive rather than growing.

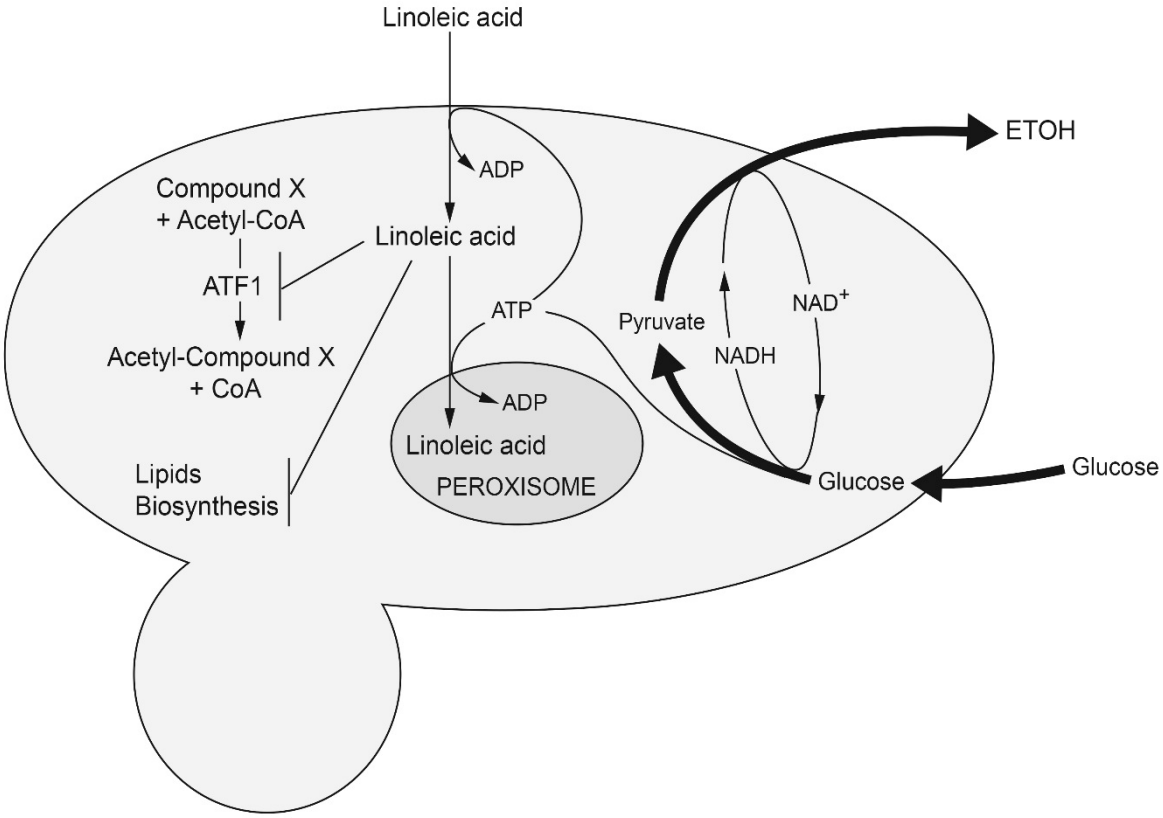

Figure 5.1. Overall effect of linoleic acid on *Saccharomyces cerevisiae* metabolism. Linoleic acid imported into *S. cerevisiae* cell requires ATP, which increases the alcoholic fermentation rate to recycle NADH generated during glycolysis (thick arrows). Once in the cell, linoleic acid is transported to the peroxisome, again in an energy-dependant process. As a consequence, lipid biosynthesis decreases affecting the overall lipid content of the cell. Moreover, linoleic acid inhibits the expression of an acetyltransferase gene (ATF1) reducing the overall cellular acetylation process.

5.3. Future remarks

5.3.1. *S. cerevisiae* strain selection for desired wine aroma production

From the first part of my study it was clear that linoleic acid has a different effect on Sauvignon blanc aroma according to the *S. cerevisiae* strain used for wine making (Casu et al., 2016). This leads us to think that specific strains could be selected to obtain the desired levels of aroma compounds by fermenting a grape juice with linoleic acid content manipulated by direct juice supplementation using food grade linoleic acid or via a juice blending technique. Thus, the wine industry could use this information to set up a series of fermentations with combinations of different strains and linoleic acid concentrations and later, select the desired combination.

5.3.2. Ethanol production improvement

My results demonstrated for the first time that linoleic acid supplementation under anaerobic conditions increases the *S. cerevisiae* production of ethanol. There is great interest around bio-ethanol production (Badwal et al., 2015) to be used to convert chemical energy into electrical energy. In fact, 90 billion litres of bio-ethanol is produced every year all over the world (Badwal et al., 2015). This study offers novel information to be potentially used to look into more efficient ways to produce ethanol. However, we tested only one *S. cerevisiae* strain, therefore other strains could also be tested to verify if they have a similar efficiency. Moreover, since my results showed that ethanol production increases with the linoleic acid concentration, higher levels of this fatty acid could be tested to find the maximum ethanol yield.

5.3.3. *S. cerevisiae* anaerobic alternative to the β-oxidative pathway

This study showed for the first time that a yeast species was able to oxidise a fatty acid without the presence of oxygen. This was a remarkable finding for its novelty and rarity. Some bacteria are known to have alternative pathways to β-oxidation without the

need of oxygen (Campbell et al., 2003; Jackson et al., 2008; McInerney et al., 1981) but so far this is the first time that it is observed in a yeast species. The questions that arise from this discovery are if other fatty acids can be degraded by *S. cerevisiae* under anaerobic conditions. This could be verified, as I did, by following the carbon-labelled fatty acid into the cell. Moreover, the actual pathway and the enzymes involved also would need investigation, for instance through gene expression and proteome analysis.

5.3.4. Effect of linoleic acid on the diet: from *S. cerevisiae* to humans

The supplementation of linoleic and conjugated linoleic acid to *S. cerevisiae* cultures decreased the total lipid content of the cells. Would linoleic acid supplementation in a human diet also reduce lipid biosynthesis? Literature suggests that dietary linoleic acid and its isomer, in particular the 9-cis,11-trans-LA (CLA) used in this study as well, have a two different effects on humans. In fact, it was observed that dietary CLA reduces the accumulation of body fat in humans, which matches with the data on *S. cerevisiae* in **Chapter 4,** reducing cancer progression, improving insulin resistance and fighting cardiovascular diseases (Fuke & Nornberg, 2016)**.** However, data on dietary linoleic acid are not as positive, probably due to the easy oxidation of linoleic acid. In fact, reducing its consumption lowers the formation of dangerous oxidised linoleic acid metabolites that have been implicated in a variety of pathological conditions (Ramsden et al., 2012), while the replacement of saturated fats with linoleic acid increased the mortality rate (Ramsden et al., 2013). Since the linoleic acid effect on *S. cerevisiae* metabolism and on humans are quite similar, yeast cells could be used as model to investigate further the mechanisms behind the effect these unsaturated fatty acids on human metabolism.

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