# Effects of Adding Unsaturated Fatty Acids on Fatty Acid Composition of *Saccharomyces cerevisiae* and Major Volatile Compounds in Wine

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**The synergistic influences of three unsaturated fatty acids (UFAs, namely linoleic acid, oleic acid and linolenic acid) on the fatty acid composition of *Saccharomyces cerevisiae* and major volatile compounds were investigated in synthetic grape juice. The addition of UFAs led to a corresponding increase in UFAs in the cellular lipid, which was accompanied by a reverse reduction in the content of medium-chain saturated fatty acids (C6:0 to C14:0) and little variation in that of long-chain saturated fatty acids (C16:0 to C24:0). The supplementation of UFAs considerably improved yeast growth and fermentation activity and, in particular, increased the concentrations of most volatile compounds in wine, including higher alcohols (2-phenylethanol, 2-methyl-1-propanol and 3-(methylthio)-1-propanol), medium-chain fatty acids (butanoic acid, hexanoic acid and octanoic acid), acetate esters (isoamyl acetate and 2-phenylethyl acetate) and all ethyl esters. Remarkable linear relationships were further found between ethyl esters and the concentration of the added UFAs ($R^2$ from 0.909 to 0.996), which significantly intensified the fruity, flowery and sweet attributes of the final wine, as assessed by calculating the odour activity values. Our results suggest that rationally increasing the concentration of UFAs is not only a practical method to improve yeast fermentation activity, but also a potential approach to manipulating wine aroma.**

## INTRODUCTION

Unsaturated fatty acids (UFAs) are required for *Saccharomyces cerevisiae* to grow under anaerobic conditions. They are essential not only for maintaining membrane integrity and function, but also for adapting to fermentation stresses, such as high sugar and ethanol toxicity (Holcberg & Margalith, 1981; You *et al.*, 2003; Rupčić & Jurešić, 2010). A higher degree of unsaturation of the cell membrane can maintain higher membrane fluidity and guarantee the activity of membrane-associated enzymes and transporters, such as ATPase and general amino acid permease (GAP) (Calderbank *et al.*, 1985; Rosa & Sá-Correia, 1992). The main UFAs of *S. cerevisiae* are oleic acid (18:1) and palmitoleic acid (16:1), which are derived from the desaturation of the saturated fatty acids (SFAs) palmitic acid (16:0) and stearic acid (18:0) by the oxygen-dependent enzyme $\Delta^9$ desaturase encoded by the *OLE1* gene respectively (Yamada *et al.*, 2005). Because it lacks $\Delta^{12}$-fatty acid desaturase and ω3-fatty acid desaturase, *S. cerevisiae* cannot produce polyunsaturated fatty acids (PUFAs), such as linoleic acid (18:2n6) and α-linolenic acid (18:3n3). The ability of PUFAs to maintain membrane fluidity is higher than that of monounsaturated fatty acids (MUFAs), and they

thus offer better protection to cells under harsh conditions (Yazawa *et al.*, 2009).

The lipid and fatty acid composition of yeast cell membranes can be modified by fermentation temperature, oxygen availability, growth rate and the presence of sterols, which in turn influence cell growth and fermentation activity (Fujii *et al.*, 1997; Redon *et al.*, 2009) and strongly affect the formation of volatile metabolites, including esters, acetate and ethyl esters, higher alcohols and medium-chain fatty acids (MCFAs), all of which are relevant to wine aroma (Thurston *et al.*, 1982; Mauricio *et al.*, 1997; Beltran *et al.*, 2008; Varela *et al.*, 2012). During wine fermentation, the absence of oxygen suppresses the fatty acid desaturation of yeast. An alternative to the biosynthesis is the direct uptake of UFAs from the grape juice, which can be used to avoid yeast stuck fermentation (Varela *et al.*, 2012).

Grape berries contain 0.15% to 0.24% (wet weight basis) lipids (Gallander & Peng, 1980). UFAs represent the major components of the total lipids. Linoleic acid (C18:2n6) is the most abundant, followed by oleic acid (C18:1n9) and α-linolenic acid (C18:3n3) (Ancín *et al.*, 1998; Santos *et al.*, 2011). The concentrations of UFAs in grape must depend largely on the grape cultivar (Ancín *et al.*, 1998) and

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*285*

technological fermentation procedures, such as grape must clarification (Varela *et al.*, 1999) and grape-skin maceration (Valero *et al.*, 1998). Several studies have investigated the effect of exogenous UFAs on intracellular fatty acid composition or wine aroma biosynthesis during wine fermentation. Redon *et al.* (2009) found that supplementation with palmitoleic acid (C16:1) can shorten the chain length and reduce total fatty acids (including UFAs and SFAs) compared with the control, which increases the viability of the yeast and reduces the duration of wine fermentation. The profile of aroma compounds was not available in this study. The influence of exogenous UFAs on aroma compounds is associated with the type and concentration of UFAs. The addition of oleic acid (31 mg/L) and ergosterol (25 mg/L) can increase the production of higher alcohols and acetate esters, except for 1-butanol and 1-pentanol, which exhibit the opposite trend (Mauricio *et al.*, 1997). Thurston *et al.* (1982) found that the presence of linoleic acid (50 mg/L) can suppress the production of ethyl esters and acetate esters. In one study of a synergistic effect, Tween 80, containing 70% of oleic acid and 30% of palmic acid and stearic acid, was added and improved the esters, higher alcohols and volatile fatty acids of wine (Varela *et al.*, 2012).

Because the three UFAs coexist in grape must and the concentrations vary with grape cultivar and fermentation procedure, it is essential from the wine production perspective to investigate their synergistic effect on the yeast fatty acid composition of yeast cells and yeast-derived aroma compounds during wine fermentation. To date, however, no completed information is available. Therefore, in this study we investigated the synergistic effects of UFAs, including linoleic acid, oleic acid and linolenic acid, on yeast fermentation activity and the fatty acid compositions of cells and volatile aroma compounds. To achieve this purpose, four series concentrations of UFA mixtures were set according to their concentration and ratio in real grape must, which were initially added to the medium (Gallander & Peng, 1980; Delfini *et al.*, 1992; Ancín *et al.*, 1998; Valero *et al.*, 1998; Varela *et al.*, 1999; Santos *et al.*, 2011). To facilitate this investigation, a simplified, chemically defined medium (MS300) that resembles the nutrient composition of grape juice was used.

MATERIALS AND METHODS
**Yeast strains and culture media**
The *S. cerevisiae* strain EC1118 (Lallemand Inc. Denmark) was used in this study. The composition of the synthetic grape must MS300 was similar to that used in previous research (Bely *et al.*, 1990), with a little modification. Because the purpose of the study was to investigate the effect of UFAs

on yeast cell metabolism, Tween 80 and sodium oleate were not added. The components in the MS300 medium were as follows: main compounds (in g/L): glucose (240), citric acid (6), malic acid (6), $KH_2PO_4$ (0.75), $K_2SO_4$ (0.5), $MgSO_4$-$7H_2O$ (0.25), $CaCl_2$-$2H_2O$ (0.155), NaCl (0.2); mineral salts (mg/L): $MnSO_4$-$H_2O$ (4), $ZnSO_4$ (4), $CuSO_4$-$5H_2O$ (1), KI (1), $CoCl_2$-$6H_2O$ (0.4), $H_3BO_3$ (1), $NaMoO_4$-$H_2O$ (1); vitamins (in mg/L): myo-inositol (20), nicotinic acid (2), calcium pantothenate (1.5), thiamine hydrochloride (0.25), pyridoxine hydrochloride (0.25), biotin (0.003); nitrogen compounds (in mg/L): proline (612.6), glutamine (505.3), arginine (374.4), tryptophan (179.3), alanine (145.3), glutamic acid (120.4), serine (78.5), threonine (759.2), leucine (48.4), aspartic (44.5), valine (44.5), phenylalanine (37.9), isoleucine (32.7), histidine (32.7), methionine (31.4), tyrosine (18.3), glycine (18.3), lysine (17), cysteine (13.1), ammonium chloride (460); lipids (in mg/L): ergosterol (15). The UFA mixture (including linoleic acid, oleic acid and *α*-linolenic acid, purchased from Sigma, purity ≥ 98%) was added to the medium in the concentrations shown in Table 1. The minimum UFAs were required to ensure normal cell growth and fermentation under anaerobic conditions (Aceituno *et al.*, 2012), which served as the control (Table 1, medium A). The concentration of UFAs in media B, C and D were four-, eight- and 12-fold higher than that in medium A respectively. The proportions of the three UFAs were determined according to the results of previous studies (Ancín *et al.*, 1998; Santos *et al.*, 2011).

**Fermentation conditions**
Yeast strains were pre-cultured aerobically in YEPD (1% yeast extract, 2% peptone, 2% glucose) at 120 rpm and 28 °C for approximately 15 h to the late exponential phase. Before the inoculums, nitrogen was sparged to eliminate the oxygen from the medium. The inoculations were in 500 mL flasks equipped with glass capillary stoppers containing 300 mL of MS300 medium. The initial yeast inoculums ($1 \times 10^6$ CFU/mL) were cultivated statically at 25 °C. Fermentation was carried out in three biological repeats. The progress of fermentation was monitored daily by measuring cell density ($OD_{600}$) and sugar consumption. Samples taken from the fermentation flasks were immediately centrifuged to collect the cell-free supernatants for the analysis of the main fermentation products and aroma compounds and the yeast cells for the analysis of intracellular fatty acids. UFA changes in the media and the intracellular fatty acids during fermentation were analysed at the pre-fermentation (0 h), mid-exponential (50 h), late-exponential (70 h), end-exponential (94 h) and mid-stationary phases (166 h), and at the end of fermentation (266 h for media A, B and C and 238 h for

TABLE 1
Concentrations of oleic acid, linoleic acid and *α*-linolenic acid in MS300 media (A, B, C and D)

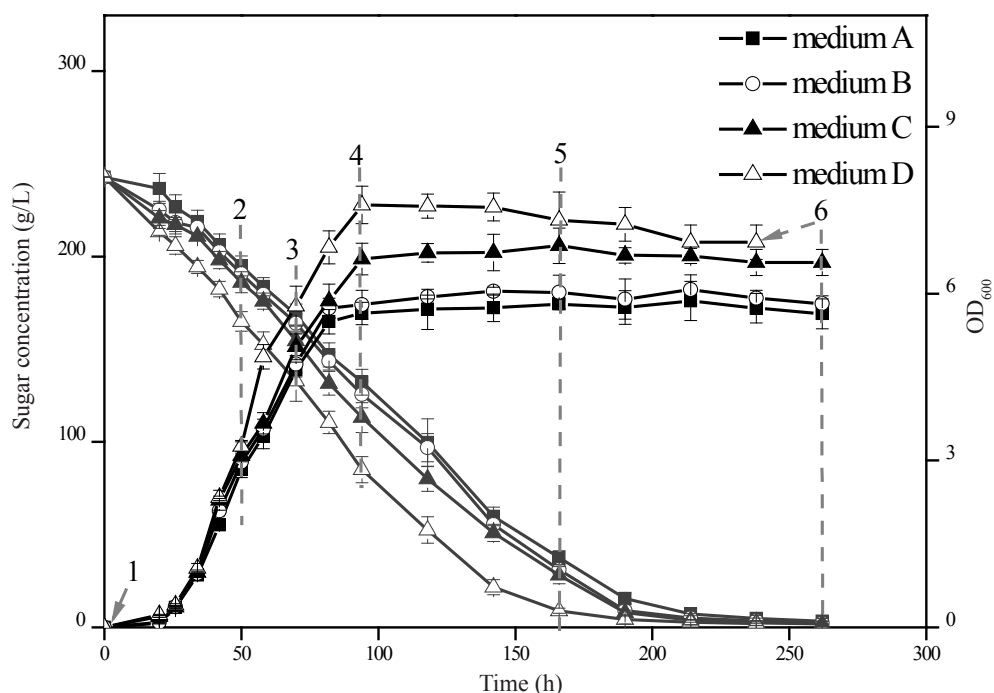| Variable | Concentrations of UFAs in different media (mg/L) | | | |
|---|---|---|---|---|
|  | medium A | medium B | medium C | medium D |
| oleic acid | 10 | 50 | 90 | 130 |
| linoleic acid | 30 | 150 | 270 | 390 |
| *α*-linolenic acid | 8 | 40 | 72 | 104 |

FIGURE 1

Cell growth (OD600) and residual sugar concentration during alcoholic fermentation in different media. Fermentation phase (fermentation time): mid-exponential (50 h), late-exponential (70 h), end-exponential (94 h) and mid-stationary phases (166 h) and at the end of fermentation (266 h for media A, B and C and 238 h for medium D).

medium D) (Fig. 1). The analysis of the main fermentation products and aroma compounds were conducted at the end of fermentation. All fermentation samples were stored at -20 °C until analysis. Each analysis was repeated in triplicate for each biological repeat.

**Analysis of main fermentation products**
The concentrations of the main fermentation products, glucose, fructose, ethanol, glycerol, malic acid, citrate acid and acetic acid, were determined by high-performance liquid chromatography (HPLC, 1200 series, Agilent Technologies, Inc., Palo Alto, CA, USA) using an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, CA, USA), as described in a previous study but with a little modification (López & Gómez, 1996). The mobile phase flow rate of 0.6 ml/min was achieved with an elution gradient composed of 5 nmol/L $H_2SO_4$ in water: (1) The injection volume was 20 μL for the glucose, fructose, glycerol and alcohol analysis. The column temperature was 45°C with a refractive index detector (RID). The total analysis time was 30 min. (2) The injection volume was 10 μL for the malic acid, citric acid and acetic acid analysis. The column temperature was 60 °C, with a diode array detector (210 nm, DAD). The total analysis time was 30 min.

**Analysis of fatty acids**
The analysis of the fatty acids in the yeast cells was conducted according to the procedure of Redon *et al.* (2009). Yeast cell samples were placed in sealed tubes and saponified with 1 mL of 5% NaOH in 50% methanol/water. The saponification was conducted at 100 °C for 30 min. The saponified part then was cooled to room temperature, and 2 mL HCl 6 M

was added to the tubes. Free fatty acids were extracted by adding 0.5 mL of hexane:methyl-tert-butyl ether (1:1, v/v). The tubes were vortexed twice for 30 sec each. The organic phase was collected after centrifugation (3 000 rpm, 3 min).

Fatty acids were quantified with a 7890NGC gas chromatograph equipped with a 5975BMS mass spectrum system (GC-MS, Agilent, USA) on an HP-INNOWAX column (60 m × 0.25 i.d., 0.25 μm film thickness, J&W Scientific, USA) under a helium carrier gas (1 ml/min). The temperature was programmed from 80 °C (held for 1 min) to 220 °C at 25 °C/min and from 220 °C to 250 °C (held for 20 min) at 5 °C/min. One microlitre of extract was injected in splitless mode. The temperature of the transfer line and the injector were 280 °C and 250 °C respectively. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV, and in the selective ion mode (SIM) range of 20 to 350 m/z. The calibration was previously performed with pure standard chemicals.

**Analysis of aroma compounds**
The analysis of the final wine odour aromas was conducted according to previous research (Zhang *et al.*, 2007), as follows. Five millilitres of the final fermentation sample, 1.00 g of NaCl and 10 μL of 4-methyl-2-pentanol (1.039 mg/mL water, internal standard) were blended in a 15 mL sample vial that was tightly capped with a PTFE silicon septum and contained a magnetic stirrer. Subsequently, the vial containing the sample was heated at 40°C for 30 min on a heating platform by agitation (80 rpm). A 7890NGC gas chromatograph equipped with a 5975BMS mass spectrum system (GC-MS, Agilent, USA) on an HP-INNOWAX column (60 m × 0.25 i.d., 0.25 μm film thickness, J&W

Scientific, USA) was applied for the analysis of the aroma compounds. The pre-treated (conditioned at 270 °C for 1 h) SPME fibre (50/30 μm DVB/Carboxen/PDMS, Supelco, Bellefonte, PA, USA) was then inserted into the headspace and extracted for 30 min with continued heating (40 °C) and agitation (80 rpm). The fibre was instantly desorbed in the GC injector for 8 min at 250 °C. The GC inlet was set in the splitless mode. The starting temperature of the oven was held at 50 °C for 1 min and then increased to 220 °C at a rate of 3 °C/min and held there for 5 min. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV, and in the scan and selective ion mode (SIM) range of 35 to 350 m/z. The calibration was previously performed with pure standard chemicals based on previous studies (Howard *et al.*, 2005).

**Establishment of odour activity value and aromatic series**
To evaluate the contribution of each aroma compound to the total odour profile, the odour activity value (OAV) was calculated by dividing the concentration of a compound by its odour threshold (OT, determined from the wine matrix) that was obtained from the literature cited in Table 4. To obtain the whole aroma profile of the wine, an aromatic series was established by grouping the OAVs of the volatiles exhibiting similar odour descriptions (Peinado *et al.*, 2006; Wu *et al.*, 2011).

**Statistical analysis**
Analysis of variance (ANOVA, least significant difference method at a significance level of $P \leq 0.05$) was used to evaluate differences between the volatile and non-volatile compounds in the fermentation samples by the SPSS statistical package version 19.0 (SPSS Inc., USA).

RESULTS AND DISCUSSION
**Yeast growth and sugar consumption**
Fig. 1 shows the progress of fermentation with different UFA concentrations. The addition of UFAs greatly improved yeast growth and sugar consumption. Cells reached the stationary phase after 100 h of fermentation in all cases. The highest biomass ($OD_{600}$ values) was observed in medium D, followed by media C, B and A. The sugar concentration in medium D was reduced below 2 g/L after 238 h of fermentation and was shortened by 28 h compared with that of the three other cases. Correspondingly, a positive relationship between initial UFA concentration and the maximum biomass ($OD_{600}$ values, $R^2 = 0.9514$) and maximum glucose consumption rates ($R^2 = 0.9567$) was established. These results confirmed the conclusion that the initial addition of UFAs can improve yeast growth and increase yeast fermentation activity (Redon *et al.*, 2009).

**Main fermentation products**
The final concentrations of ethanol, glycerol, acetic acid, citric acid and malic acid were further determined, and the results show that there was no significant difference among the different samples (Table 2). This finding suggests that the variation in the UFA concentrations did not have a considerable influence on the primary metabolites of the sugar.

**Fatty acid composition of yeast cells**
It generally is believed that the improvement in cell growth and fermentation performance that is caused by UFAs results from the modification of the composition of intracellular fatty acids. We thus determined the fatty acid content of the cells. First, the profiles of the supplemented UFA concentrations in the media during fermentation were investigated. The results showed that the three UFAs were rapidly taken up by yeast cells and that the UFA levels decreased rapidly. The highest UFA uptake rates were observed in medium D during fermentation, followed by media C, B and A (Fig. 2). In four cases, the UFAs were almost completely consumed after 94 h of fermentation when the yeast cells reached the end-exponential phase (data not shown). Correspondingly, the intracellular UFA content increased significantly and the level of particular UFAs was positive following the supplemented UFA concentration (Fig. 3a). The incorporation of UFAs led to an increase in UFAs in the total fatty acid content from 74.35% in medium A to 80.55% in medium C and 83.55% in medium D when the cells reached the mid-exponential phase; the highest UFA levels were obtained at this time. After the mid-exponential phase, the UFA content gradually decreased and finally was maintained at a constant level after the end-exponential phase in all samples, but the values with UFAs addition were all higher than those of the control (medium A). Our results were consistent with a previous study, which showed that the UFA concentration of yeast cells declined as the fermentation progressed due to cell growth under anaerobic fermentation (Venter *et al.*, 1989). The incorporation of UFAs resulted in the highest total concentration of FAs observed in medium D, followed by yeast in media C, B and A (Fig. 3d, ANOVA, P < 0.05).

It should be noted that palmitoleic acid (C16:1n-9), which is considered one of the main fatty acids in the composition of yeast cells, was not detected in our research (data not shown). This can be ascribed to the fact that fatty acid desaturation cannot occur in yeast cells under anaerobic conditions (Snoek & Steensma, 2007). Our results demonstrate that the enrichment of UFAs in cells is essential for yeast adaptation to environmental stress during alcoholic fermentation (You *et al.*, 2003; Rupčić & Jurešić, 2010).

Long-chain saturated fatty acids (LSFAs, C16:0 to

TABLE 2
Main fermentation products of the final wines

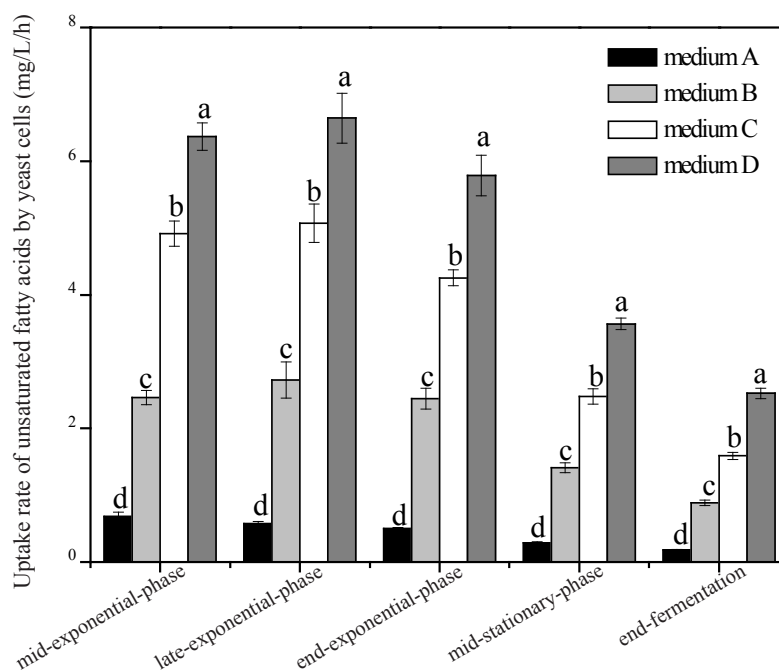| Medium | ethanol (%, v/v) | glycerol (g/L) | acetic acid (g/L) | citrate acid (g/L) | malic acid (g/L) |
|--------|------------------|----------------|-------------------|--------------------|------------------|
| A | 11.46 ± 0.22 | 6.08 ± 0.08 | 0.65 ± 0.04 | 4.73 ± 0.12 | 6.07 ± 0.01 |
| B | 10.93 ± 0.37 | 5.99 ± 0.07 | 0.66 ± 0.03 | 4.62 ± 0.10 | 5.90 ± 0.19 |
| C | 11.45 ± 0.16 | 6.31 ± 0.04 | 0.73 ± 0.04 | 4.78 ± 0.02 | 6.08 ± 0.03 |
| D | 11.18 ± 0.13 | 6.17 ± 0.03 | 0.72 ± 0.02 | 4.68 ± 0.08 | 6.08 ± 0.12 |

FIGURE 2

Uptake rates of unsaturated fatty acids (UFAs) by yeast cells in different media during alcoholic fermentation. Significant differences in UFA concentrations among media in the same fermentation phase are labelled by letters a, b, c and d (ANOVA, $P < 0.05$).
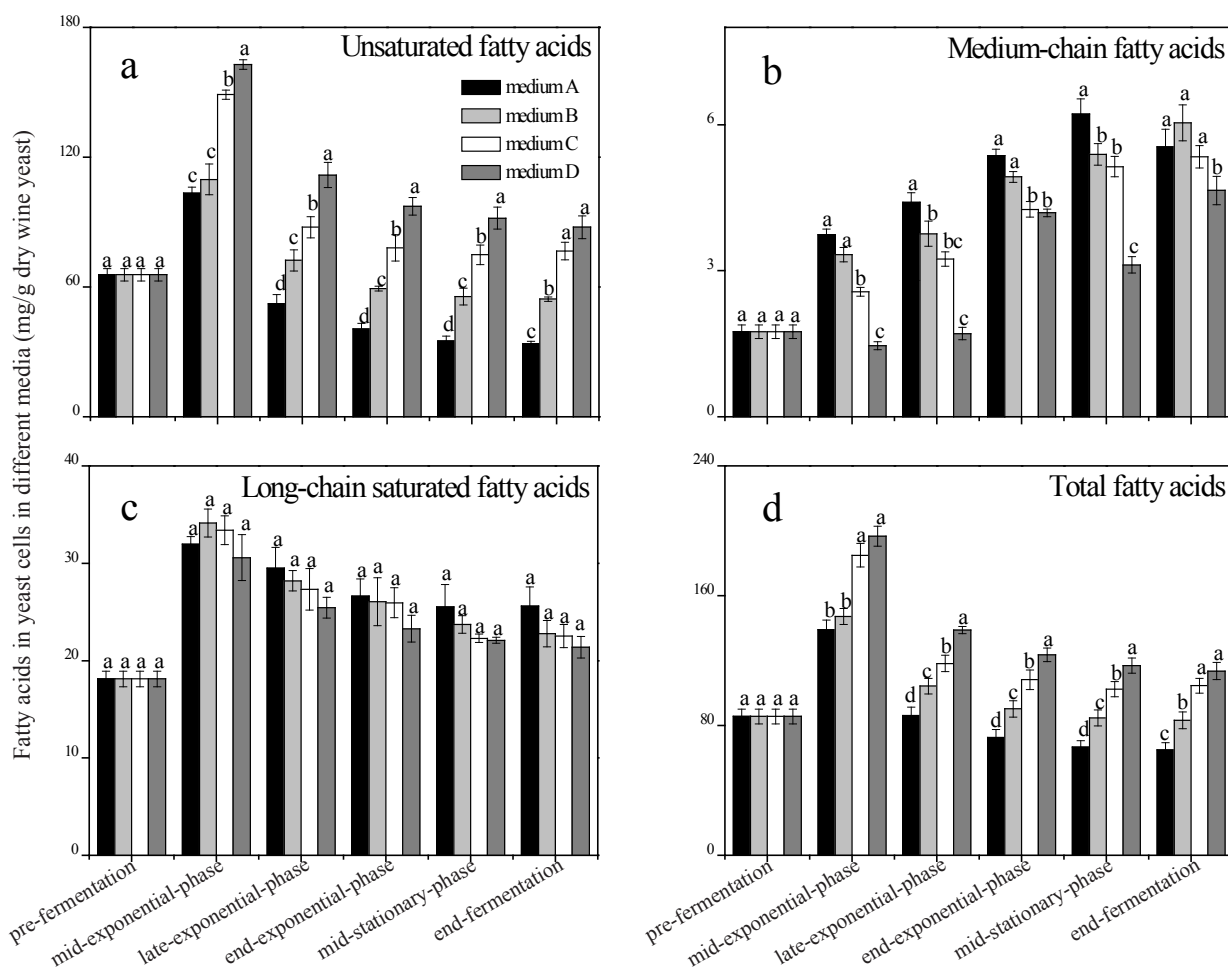


FIGURE 3

Concentrations of unsaturated fatty acids (UFAs), medium-chain fatty acids (MCFAs), long-chain saturated fatty acids (LCSFAs) and total fatty acids (TFAs) in wine yeast during wine fermentation. Significant differences in main fatty acid concentrations among yeast in different wines in the same fermentation phase are labelled by letters a, b, c and d (ANOVA, $P < 0.05$).

C24:0, 15.54% to 39.38%) were the second largest group of fatty acids in the total fatty acids, followed by medium-chain fatty acids (MCFAs C6:0 to C14:0, 0.74% to 9.32%). Surprisingly, the variation in intracellular UFA concentration had no significant effect on LSFA synthesis, but it did have a considerable impact on the content of MCFAs. The highest concentration of MCFAs was observed in yeast cultivated in medium A, followed by media B, C and D (Fig. 3b, ANOVA, $P < 0.05$), which suggests that there is a negative relationship between the intracellular MCFAs and UFAs. At present we cannot fully explain the phenomenon; however, two deductions were proposed according to previous studies. First, Dufour *et al*. (2008) suggested that the increase in UFAs in yeast cells can lead to the release of MCFAs from the fatty acid synthase complex (FAS1 and FAS2) and further secrete them into the medium. This phenomenon was also observed in our study, as shown below. The secretion might be partially responsible for the reduction in MCFA content in the cells. An alternative explanation, proposed by Redon *et al*. (2011), states that, in addition to UFAs, MCFAs are functional compounds that help yeast cells adapt to stress conditions by increasing the membrane fluidity. The incorporation of exogenous UFAs into the cell membrane might be efficient for cells to handle stress conditions and may thus weaken the synthesis of MCFAs. The detailed mechanism needs to be investigated further.

**Aroma compounds**
Although they had no considerable impact on the primary metabolic compounds of sugar, the variations in UFAs significantly affected the synthesis of wine aroma compounds. Generally, the volatile compounds were promoted by the addition of UFAs, and the total concentrations in the final wines of C and D were 1.21- and 1.25-fold that observed in wine A respectively (Table 3, ANOVA, $P < 0.05$). Moreover, the concentrations of ethyl esters, which are the main aroma-contributing compounds, in wines C and D were 1.84- and 2.82-fold higher than that in wine A respectively. Twenty-eight aroma compounds were detected in our study – six MCFAs, seven alcohols, five aldehydes, seven ethyl esters and three acetate esters. Of these, 22 compounds could be identified, and six compounds could be identified tentatively (Table 3). With the exception of undecyl aldehyde in wine D, all of the aroma compounds could be quantified in all the samples.

*Medium-chain fatty acids*
MCFAs (C6 to C12) are produced primarily by yeasts as intermediates in the biosynthesis of long-chain fatty acids and are prematurely released from the fatty acid synthase (FAS) complex. These volatile fatty acids can directly contribute to the flavour of wine, or serve as substrates that participate in the formation of ethyl acetates. In contrast to the intracellular MCFAs, the addition of UFAs resulted in a greater accumulation of MCFAs in the medium. This result can be ascribed to the repression of the gene encoding acetyl-CoA carboxylase (ACC1) by exogenous UFAs, which causes the release of MCFAs from the fatty acid synthase complex (FAS1 and FAS2) and their secretion into the environment (Dufour *et al*., 2008). Among the six identified MCFAs,

octanoic acid was the highest, followed by hexanoic acid, decanoic acid, dodecanoic acid, butanoic acid and heptanoic acid. The highest concentrations of octanoic acid, hexanoic acid and butanoic acid were found in wine C (14.59, 3.26 and 0.35 mg/L) and wine D (12.44, 3.23 and 0.36 mg/L), compared with those of wine A (10.22, 2.21 and 0.27 mg/L) (Table 3, ANOVA, $P < 0.05$).

*Ethyl esters*
The C6, C8 and C10 medium-chain fatty acid ethyl esters are important flavour-active metabolites that are responsible for the highly desired fruit aroma characters in wine. They are generated by an alcohol and the acyl group derived from activated MCFAs. Among the seven identified ethyl esters, ethyl decanoate was quantitatively the highest, followed by ethyl octanoate, ethyl hexanoate and ethyl dodecanoate. Higher contents of ethyl esters were formed in wines C and D, and the total concentrations were 1.84- and 2.82-fold higher than that in wine A (Table 3, ANOVA, $P < 0.05$) respectively. In addition, significant positive relationships between ethyl esters, particularly ethyl hexanoate ('fruity' odour) and ethyl octanoate ('fruity', 'floral' and 'sweet' odour), and the added UFA concentrations were established ($R^2$ from 0.909 to 0.996). Sufficient MCFAs are crucial for the higher biosynthesis of pleasant ethyl esters (Saerens *et al*., 2008); therefore, the improvement observed for the ethyl esters following fatty acid treatment by UFAs can be ascribed to the accumulation of the substrate MCFAs in the medium. This conclusion was supported further by the observation that linear correlations existed between MCFAs and the corresponding ethyl esters (data not shown).

*Alcohols*
Seven alcohols were identified in our research, including three fatty alcohols (1-hexanol, 1-heptanol and 1-octanol) and four higher alcohols (2-phenylethanol, 3-methyl-1-butanol, 2-methyl-1-propanol and 3-(methylthio)-1-propanol). The detailed mechanism of fatty alcohol biosynthesis by wine yeast is not fully understood. Our results indicate that treatment with UFAs can increase the production of fatty alcohols. The highest concentrations of 1-hexanol and 1-octanol were found in wine D (444.60 μg/L and 108.24 μg/L *vs* 35.93 and 12.93 μg/L in wine A respectively), whereas the highest concentration of 1-heptanol was in wine C (42.82 μg/L *vs* 28.14 μg/L in wine A) (Table 3, ANOVA, $P < 0.05$).

The higher alcohols are predominantly formed by yeast during fermentation from $\alpha$-keto acids, a process that involves the degradation of an amino acid (*i.e.*, valine, leucine, isoleucine, threonine and phenylalanine) via the so-called Ehrlich pathway (Hazelwood *et al*., 2008). High levels of 2-phenylethanol, 2-methyl-1-propanol and 3-(methylthio)-1-propanol were observed in wines C and D. Higher alcohols can have both a positive and negative impact on the aroma and flavour of wine, depending on their concentrations. Excessive concentrations (exceeding 400 mg/L) can result in a strong, pungent smell, whereas an optimal level (below 300 mg/L) imparts a pleasant character (Bartowsky & Pretorius, 2009). In this study, total concentrations of 295.9 mg/L and 306.3 mg/L were found

TABLE 3
Concentrations of aroma compounds in wines

| Aroma compounds | Odour threshold | Retention index | ID | Fermentation medium | | | |
|---|---|---|---|---|---|---|---|
| | | | | A | B | C | D |
| butanoic acid (mg/L) | 1 (a) | 1630 | I | $0.27 \pm 0.01^b$ | $0.27 \pm 0.03^b$ | $0.35 \pm 0.03^a$ | $0.36 \pm 0.04^a$ |
| hexanoic acid (mg/L) | 0.42 (b) | 1855 | I | $2.21 \pm 0.15^b$ | $2.66 \pm 0.13^{ab}$ | **$3.26 \pm 0.29^a$** | **$3.23 \pm 0.33^a$** |
| heptanoic acid (µg/L) | | 1990 | I | $62.44 \pm 5.77^a$ | $75.09 \pm 7.59^a$ | $82.78 \pm 1.62^a$ | $77.85 \pm 5.68^a$ |
| octanoic acid (mg/L) | 0.5 (b) | 2075 | I | **$10.22 \pm 0.6^b$** | **$13.16 \pm 1.58^a$** | **$14.54 \pm 1.59^a$** | **$12.44 \pm 1.45^a$** |
| decanoic acid (mg/L) | 1 (b) | 2292 | I | $2.37 \pm 0.38^a$ | $2.41 \pm 0.48^a$ | $2.8 \pm 0.36^a$ | $2.29 \pm 0.47^a$ |
| dodecanoic acid (mg/L) | 1 (b) | 2500 | I | $1.29 \pm 0.17^a$ | $1.37 \pm 0.17^a$ | $1.55 \pm 0.14^a$ | $1.71 \pm 0.15^a$ |
| Total medium-chain fatty acids (mg/L) | | | | $16.42 \pm 1.43^b$ | $19.94 \pm 1.02^a$ | $22.58 \pm 2.13^a$ | $20.11 \pm 1.87^a$ |
| ethyl hexanoate (mg/L) | 0.005 (a) | 1232 | I | **$0.22 \pm 0.02^c$** | **$0.25 \pm 0.05^c$** | **$0.98 \pm 0.1^b$** | **$1.57 \pm 0.1^a$** |
| ethyl octanoate (mg/L) | 0.002 (a) | 1429 | I | **$0.69 \pm 0.05^b$** | **$0.71 \pm 0.02^b$** | **$0.88 \pm 0.12^a$** | **$1.01 \pm 0.16^a$** |
| ethyl nonanoate (µg/L) | | 1535 | I | $0.65 \pm 0.18^d$ | $1.1 \pm 0.09^c$ | $1.88 \pm 0.14^b$ | $2.86 \pm 0.18^a$ |
| ethyl decanoate (mg/L) | 0.2 (a) | 1637 | I | **$0.9 \pm 0.2^d$** | **$1.64 \pm 0.1^c$** | **$3.14 \pm 0.33^b$** | **$4.13 \pm 0.43^a$** |
| ethyl dodecanoate (mg/L) | 2 (c) | 1848 | I | $0.11 \pm 0.01^d$ | $0.25 \pm 0.03^c$ | $0.44 \pm 0.05^b$ | $0.61 \pm 0.01^a$ |
| ethyl myristate (µg/L) | 2 000 (c) | 2055 | TI | $4.92 \pm 0.52^c$ | $11.74 \pm 1.55^b$ | $21 \pm 2.18^a$ | $27.65 \pm 4.88^a$ |
| ethyl palmitate (µg/L) | > 2 000(c) | 2263 | TI | $6.52 \pm 0.61^c$ | $11.74 \pm 1.07^b$ | $17.81 \pm 1.88^a$ | $18.56 \pm 1.17^a$ |
| Total ethyl esters(mg/L) | | | | $1.93 \pm 0.16^d$ | $2.88 \pm 0.21^c$ | $5.48 \pm 0.37^b$ | $7.37 \pm 0.61^a$ |
| 1-hexanol (µg/L) | 8 000 (a) | 1344 | I | $35.93 \pm 1.7^d$ | $142.99 \pm 17.16^c$ | $231.04 \pm 15.6^b$ | $444.6 \pm 45.23^a$ |
| 1-heptanol (µg/L) | 3 (d) | 1457 | I | **$28.14 \pm 2.75^b$** | **$27.76 \pm 0.72^b$** | **$42.82 \pm 2.75^a$** | **$37.52 \pm 3.55^a$** |
| 1-octanol (µg/L) | 110 (d) | 1562 | I | $12.93 \pm 1.07^d$ | $24.92 \pm 1.17^c$ | $41.36 \pm 4.8^b$ | $108.24 \pm 16.58^a$ |
| 2-phenylethanol (mg/L) | 10 (a) | 1928 | TI | **$42.98 \pm 4.02^c$** | **$58.5 \pm 5.4^b$** | **$76.77 \pm 5.86^a$** | **$71.33 \pm 4.49^a$** |
| 3-methyl-1-butanol (mg/L) | 30 (a) | 1206 | I | **$184.21 \pm 13.45^a$** | **$157.36 \pm 22.61^a$** | **$186.51 \pm 13.62^a$** | **$205.84 \pm 24.18^a$** |
| 2-methyl-1-propanol (mg/L) | 40 (a) | 1111 | I | $18.22 \pm 1.32^b$ | $22.17 \pm 3.03^{ab}$ | $32.24 \pm 3.08^a$ | $28.55 \pm 4.4^a$ |
| 3-(methylthio)-1-propanol (µg/L) | 500 (a) | 1726 | I | $23.78 \pm 1.09^b$ | $26.88 \pm 2.83^{ab}$ | $31.74 \pm 3.6^a$ | $29.26 \pm 2.52^a$ |
| Total alcohols (mg/L) | | | | $245.5 \pm 10.10^b$ | $238.26 \pm 12.64^b$ | $295.87 \pm 14.57^a$ | $306.34 \pm 33.07^a$ |
| 3-methyl-1-butanol acetate (mg/L) | 0.03 (a) | 1022 | TI | **$1.09 \pm 0.06^b$** | **$1.25 \pm 0.15^a$** | **$1.22 \pm 0.14^a$** | **$1.69 \pm 0.16^a$** |
| ethyl acetate (mg/L) | 7.5 (a) | 877 | I | **$36.54 \pm 3.43^a$** | **$41.08 \pm 2.24^a$** | **$39.19 \pm 5.83^a$** | **$42.56 \pm 6.41^a$** |
| 2-phenylethyl acetate (µg/L) | 250 (a) | 1830 | I | $1.2 \pm 0.12^b$ | $1.75 \pm 0.15^a$ | $1.91 \pm 0.11^a$ | $1.98 \pm 0.13^a$ |
| Total acetate esters (mg/L) | | | | $37.64 \pm 3.91^a$ | $42.33 \pm 2.27^a$ | $40.41 \pm 4.24^a$ | $44.25 \pm 6.21^a$ |
| octanal (µg/L) | 0.7 (e) | 1290 | I | **$7.53 \pm 0.7^a$** | **$8.11 \pm 0.83^a$** | **$8.71 \pm 0.88^a$** | **$7.53 \pm 0.1^a$** |
| nonanal (µg/L) | 1 (e) | 1394 | I | **$8.35 \pm 1.58^c$** | **$30.51 \pm 2.43^a$** | **$34.76 \pm 4.4^a$** | **$19.93 \pm 1.75^b$** |
| decanal (µg/L) | 0.1 (e) | 1495 | I | **$1.07 \pm 0.17^c$** | **$4.99 \pm 0.25^a$** | **$5.68 \pm 0.21^a$** | **$2.84 \pm 0.11^b$** |
| undecyl aldehyde (µg/L) | | 1596 | TI | $1.43 \pm 0.14$ | $1.71 \pm 0.17$ | $1.61 \pm 0.16$ | Tr |
| dodecanal (µg/L) | 2 (f) | 1693 | TI | **$2.51 \pm 0.27^c$** | **$4.3 \pm 0.07^b$** | **$5.44 \pm 0.3^a$** | **$3.95 \pm 0.3^b$** |
| Total aldehydes (µg/L) | | | | $20.89 \pm 0.94^c$ | $94.57 \pm 6.53^a$ | $107.39 \pm 9.43^a$ | $59.87 \pm 5.21^b$ |
| Total aroma compounds concentrations (mg/L) | | | | $301.51 \pm 15.63^b$ | $303.5 \pm 24.81^b$ | $364.45 \pm 20.79^a$ | $378.13 \pm 22.91^a$ |

Odour thresholds of aroma compounds were according to previous references: (a) Guth, 1997; (b) Li *et al.*, 2008; (c) Leffingwell & Associates, 1999; (d) Fazzalari, 1978; (e),Ferreira *et al.*, 2004; (f) Boelens & Van Gemert, 1987.

ID, reliability of the identification proposal: I, identified, mass spectrum and retention index agree with standards; TI, tentatively identified, mass spectrum agrees with retention index (Wu *et al.*, 2011) and/or the mass spectral database.

Letter a, b, c, and d in the same row show significant differences according to the analysis of variance at P < 0.05. Boldface indicates concentration of aroma compound was over its odour threshold. Tr: trace.

TABLE 4

Odour active values, odour description and aromatic series of all aroma compounds in final wines

| Aroma compounds | Odour active values(OAVs) | | | | Odour description | Aromatic series |
|---|---|---|---|---|---|---|
| | A | B | C | D | | |
| hexanoic acid | $5.26 \pm 0.36^b$ | $6.33 \pm 0.31^{ab}$ | $7.76 \pm 0.69^a$ | $7.69 \pm 0.79^a$ | fatty, cheese, rancid (a) | Fatty |
| octanoic acid | $20.44 \pm 1.2^b$ | $26.32 \pm 3.16^a$ | $29.08 \pm 3.18^a$ | $24.88 \pm 2.9^a$ | fatty, rancid, harsh, cheese (a) | Fatty |
| decanoic acid | $2.37 \pm 0.38^a$ | $2.41 \pm 0.48^a$ | $2.8 \pm 0.36^a$ | $2.29 \pm 0.47^a$ | rancid, cheese (a) | Fatty |
| dodecanoic acid | $1.29 \pm 0.17^a$ | $1.37 \pm 0.17^a$ | $1.55 \pm 0.14^a$ | $1.71 \pm 0.15^a$ | laurel oil (b) | Fatty |
| ethyl hexanoate | $44.01 \pm 4^c$ | $50.02 \pm 10^c$ | $196.56 \pm 9^b$ | $314.38 \pm 20^a$ | fruity, green apple, banana (a) | Fruity |
| ethyl octanoate | $346.08 \pm 17.5^c$ | $355.19 \pm 1^c$ | $440.95 \pm 11^b$ | $506.48 \pm 10.5^a$ | sweet, floral, fruity, banana, pear (a) | Sweet, floral, fruity |
| ethyl decanoate | $4.48 \pm 1^d$ | $8.2 \pm 0.5^c$ | $15.72 \pm 1.65^b$ | $20.67 \pm 2.15^a$ | fruity, pleasant (b) | Fruity |
| octanal | $10.76 \pm 1^a$ | $11.58 \pm 1.19^a$ | $12.45 \pm 1.26^a$ | $10.76 \pm 0.14^a$ | citrus (c) | Fruity |
| nonanal | $8.35 \pm 1.58^c$ | $30.51 \pm 2.43^a$ | $34.76 \pm 4.4^a$ | $19.93 \pm 1.75^b$ | citrus (c) | Fruity |
| decanal | $10.7 \pm 1.7^c$ | $49.9 \pm 2.5^a$ | $56.8 \pm 1.1^a$ | $28.4 \pm 1.1^b$ | green, fresh (c) | Herbaceous |
| dodecanal | $1.26 \pm 0.14^c$ | $2.15 \pm 0.04^b$ | $2.72 \pm 0.15^a$ | $1.98 \pm 0.15^b$ | green coriander (c) | Herbaceous |
| 1-heptanol | $9.38 \pm 0.92^b$ | $9.25 \pm 0.24^b$ | $14.27 \pm 0.92^a$ | $12.51 \pm 0.18^a$ | green (d) | Herbaceous |
| 3-methyl-1-butanol | $6.14 \pm 0.4^a$ | $5.25 \pm 0.54^a$ | $6.22 \pm 0.59^a$ | $6.86 \pm 0.45^a$ | alcohol, nail polish (a) | Chemical |
| 2-phenylethanol | $4.3 \pm 0.35^c$ | $5.85 \pm 0.25^b$ | $7.68 \pm 0.25^a$ | $7.13 \pm 0.41^a$ | pollen, roses (e) | Floral |
| 3-methyl-1-butanol acetate | $36.47 \pm 2^b$ | $41.77 \pm 5^b$ | $40.74 \pm 4.67^b$ | $56.25 \pm 5.33^a$ | banana, fruity, sweet (a) | Fruity, sweet |
| ethyl acetate | $4.87 \pm 0.46^a$ | $5.48 \pm 0.3^a$ | $5.23 \pm 0.78^a$ | $5.67 \pm 0.85^a$ | pineapple, varnish, balsamic (e) | Fruity, chemical |

Odour description was according to the previous references: (a) Peinado *et al.*, 2006; (b) Li *et al.*, 2008; (c) Buettner *et al.*, 2003; (d) Yang *et al.*, 2008; (e) Aznar *et al.*, 2001. Significant differences in OAVs in the same row are labelled by letters a, b, c and d (ANOVA, P < 0.05).

in wines C and D respectively, which suggests that higher alcohols contributed positively to the aroma of the wine. At present, we cannot fully explain why the addition of exogenous UFAs promotes higher alcohol synthesis, but we inferred that it might be associated with the improvement of amino acid transportation into cells because it has been demonstrated that *S. cerevisiae* growing in medium enriched with UFAs (linoleic acid and oleic acid) can induce general amino acid permease (GAP) activity and increase the trans-shipment of amino acids into yeast cells (Calderbank *et al.*, 1984, 1985), which is beneficial for the synthesis of higher alcohols (Hernández-Orte *et al.*, 2006). The detailed mechanism needs to be investigated further.

*Acetate esters*
Acetate esters are another important aroma compound that are generated by the reaction of acetyl-CoA with higher alcohols that are formed from the degradation of amino acids or carbohydrates and have a significant effect on the fruity flavour of wine. The acetate esters detected in our study included ethyl acetate ('pineapple', 'varnish' and 'balsamic' odour, Table 3), isoamyl acetate ('banana' and 'sweet' odour, Table 3) and 2-phenylethyl acetate ('honey', 'roses' and 'flowery' odour). No significant difference was found in ethyl acetate in all the samples, although higher concentrations of 3-methyl-1-butanol acetate (isoamyl acetate) and 2-phenylethyl acetate accumulated in wines C

and D (Table 3, ANOVA, p < 0.05). This was in contrast to the results of a previous study, which showed that acetate esters were inhibited by exogenous UFAs due to the repression of *ATF1* transcription, the gene encoding the enzyme (alcohol acetyltransferase) that catalyses the synthesis of acetate esters (Fujii *et al.*, 1997). Our results indicate that, despite the negative regulation, increased UFA concentrations can enhance the production of acetate esters by increasing yeast growth and substrate production (higher alcohol).

*Aldehydes*
High levels of decanal and dodecanal can cause an unpleasant, intense, 'green' odour in wine, whereas a lower content of C8 to C11 aldehydes can contribute to a citrus fruit aroma (Culleré *et al.*, 2011). The effects of UFAs on the production of aldehydes were different from other aroma compounds, which were largely dependent on the UFA concentration. Relatively high UFA contents (wines B and C) resulted in higher concentrations of nonanal, decanal and dodecanal compared with wine A, whereas further increasing the UFA content (wine D) led to a great reduction in the aldehyde level (Table 3, ANOVA, P < 0.05). The OAVs decreased from 34.8-, 56.8- and 2.7-fold in wine C to 19.9-, 28.4- and 2.0-fold in wine D respectively, as shown below. This finding implies that manipulating the initial UFA concentration potentially can regulate the influence of aldehydes on wine aroma.

**Influence of adding UFAs on odour profile of final wine**

The OAV can assess the extent of the potential sensory aroma contribution for each volatile compound. An aroma compound with an OAV over one is considered to make a flavour contribution to wine (Guth, 1997). As shown in Table 4, 16 aroma compounds had an OAV above 1 in our study. According to previous research (Aznar *et al*., 2001; Buettner *et al*., 2003; Peinado *et al*., 2006; Li *et al*., 2008; Yang *et al*., 2008), these compounds are mainly associated with the attributes 'banana', 'apple', 'pear', 'strawberry', 'citrus' 'sweet', 'roses', 'fruity', 'fatty', 'cheese', 'rancid', 'nail polish', 'balsamic' and 'green' (Table 4). The OVAs of ethyl hexanoate (fruity, green apple, banana attributes) and ethyl decanoate (fruity attribute) in wine D were 6.15- and 3.62-fold higher than those of wine A. Other important volatile aroma compounds, including ethyl octanoate, 2-phenylethanol and 3-methyl-1-butanol acetate, also had higher OAVs in wines C and D than in wine A. They certainly are responsible for the increment of fruity and flowery attributes in wines C and D, as shown below.

The odour of a compound is described in terms of several descriptors that are agreed upon by experts, and an aromatic series can be defined as a group of volatile compounds with similar odour descriptors (Wu *et al*., 2011). To better understand the influence of UFAs on the wine odour profile and the contribution of various volatile compounds to the olfactory impression of wine, an aromatic series was further established in our study by combining the OAVs of a group of volatiles with similar odour descriptions (Peinado *et al*., 2006; Wu *et al*., 2011). Six aromatic series of volatile compounds were established: fruity, floral, sweet, herbaceous,

chemical and fatty (Fig. 4). Of these, the fruity series was prominent, followed by the flowery, sweet, herbaceous, fatty and chemical series. The higher OAVs of ethyl esters, higher alcohol and acetate esters (such as ethyl octanoate, ethyl hexanoate, 2-phenylethanol and 3-methyl-1-butanol acetate) led to wines C and D featuring the fruity, flowery and sweet series (Fig. 4, ANOVA, $P < 0.05$). Although wines C and D also had relatively higher OAVs in the herbaceous and fatty series, their influence on the total odour profile was limited due to the low concentration or high odour threshold of a particular compound, such as dodecanal, 1-heptanol and 1-hexanol (Table 3). As a result, there was no considerable discrepancy between the herbaceous and fatty series among the wines. Similarly, no significant difference was observed among the samples for the chemical series. Based on these results, it can be concluded that an increased initial UFA concentration in the medium can intensify the fruity, floral and sweet attributes of wine and thus improve the odour profile of the wine.

The purpose of the present study was to evaluate the synergistic impact of three UFAs on volatile compounds from the point of view of practical production. Clearly, to fully understand the mechanism of how UFAs regulate aroma compounds it is essential to investigate the separate influence of individual UFAs. In addition, the influence of yeast strain specificity should be considered. These related experiments are being conducted in our laboratory. In this study, we used the synthetic grape juice MS300 medium to eliminate the influence of grape cultivar aroma compounds and aroma precursors on the production of yeast-derived aroma compounds; however, the utilisation of natural grape must may change the effect of the variation in UFA
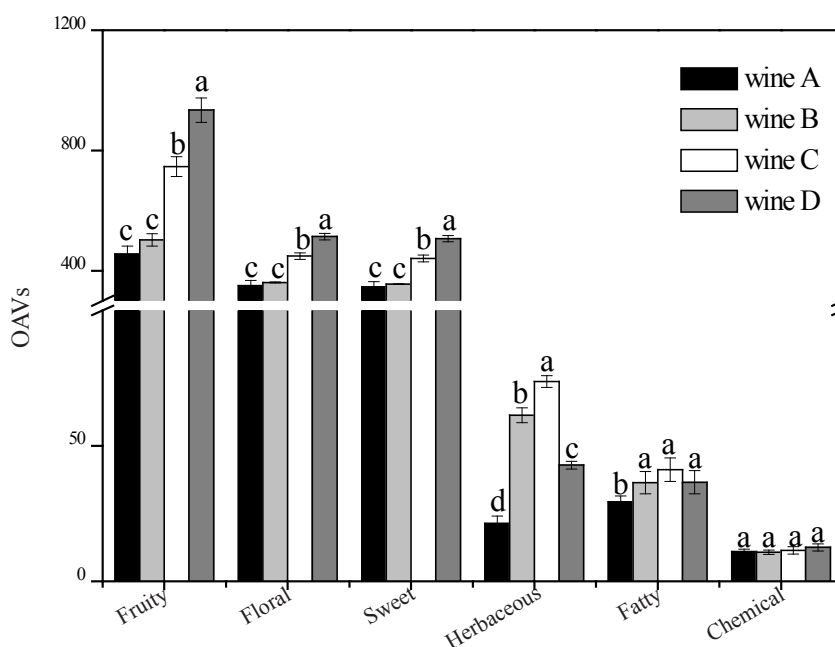


FIGURE 4

Aroma series in final wine A, wine B, wine C, and wine D. Aroma compounds are calculated for floral series: ethyl octanoate and 2-phenylethanol; for fruity series: ethyl octanoate, ethyl hexanoate, ethyl decanoate, octanal, nonanal and 3-methyl-1-butanol acetate; for sweet series: ethyl octanoate and 3-methyl-1-butanol acetate; for herbaceous series: decanal, dodecanal and 1-heptanol; for chemical series: 3-methyl-1-butanol and ethyl acetate; for fatty series: hexanoic acid and octanoic acid.

Significant difference in aroma series among wines are labelled by letters a, b, c, and d (ANOVA, $P < 0.05$).

concentration on the types and levels of volatile compounds, because complex flavour precursors (such as glycoside aroma molecules) (Loscos *et al.*, 2007) and other enzyme-catalysed reactions (such as a 1ipoxygenase-catalysed reactions) (Deluc *et al.*, 2009) may occur. Therefore, further work is needed to evaluate the extent to which changing the initial UFA concentration affects the production of wine aroma compounds in real grape must.

## CONCLUSIONS

The present results indicate that supplementation with mixed UFAs (including linoleic acid, oleic acid and linolenic acid) can correspondingly increase the intracellular UFA content, which simultaneously leads to the reverse reduction of MCFA content but does not influence LSFA synthesis. In addition to improving yeast cell growth and fermentation activity, the additions of mixed UFAs promoted the most volatile aroma compounds, including MCFAs, higher alcohols, acetate esters and all ethyl esters. A linear relationship between UFA concentrations and all ethyl esters was established, which significantly intensified the fruity, floral and sweet attributes of the final wine. Our results suggest that rationally increasing the UFA concentration in grape must could not only improve yeast fermentation activity, but also manipulate wine aroma.

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