Food Research International 123 (2019) 559–566

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Enhancing wine ester biosynthesis in mixed *Hanseniaspora uvarum*/*Saccharomyces cerevisiae* fermentation by nitrogen nutrient addition

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ARTICLE INFO

Chemical compounds studied in this article:
Ethyl acetate (PubChem CID: 8857)
Isobutyl acetate (PubChem CID: 8038)
Isoamyl acetate (PubChem CID: 31276)
Phenethyl acetate (PubChem CID: 7654)
Ethyl hexanoate (PubChem CID: 31265)
Ethyl octanoate (PubChem CID: 7799)
Ethyl decanoate (PubChem CID: 8048)

Keywords:
Flavour
Mixed culture
Yeast assimilable nitrogen
Alcoholic fermentation
Non-*Saccharomyces*
Saccharomyces cerevisiae

ABSTRACT

The dynamic changes of wine ester production during mixed fermentation with *Hanseniaspora uvarum* Yun268 and *Saccharomyces cerevisiae* F5 was investigated at different levels and timings of nitrogen nutrient addition. Nitrogen additions were performed by supplementing yeast assimilable nitrogen (YAN) into a synthetic grape must with defined composition. Ester precursors and extracellular metabolites involved in ester synthesis were analyzed throughout the fermentation. Results showed that nitrogen additions covering 50–200 mg/L YAN at the point of yeast inoculation slightly affected yeast competition and ester profiles. Interestingly, when YAN was supplemented in the mid-stage, the survival of *H. uvarum* Yun268 was enhanced, resulting in more than a 2-fold increase in the levels of higher alcohol acetates compared to that at the initial stage. Furthermore, carbon fluxes may be redistributed in the central pathway, which contributed to the production of medium-chain fatty acids and eventually triggered a 1.2-fold elevation in corresponding ethyl ester levels.

1. Introduction

Within the last decade, mixed fermentation of non-*Saccharomyces* and *Saccharomyces cerevisiae* yeasts has received a growing interest in winemaking industry due to great potentials in improving flavour component of fermented beverages such as wine, beer, and spirits (Varela, 2016). When creating and conducting mixed fermentation of these products, esters are considered as one of the most targeted components because of their olfactory contribution to desired fruity and floral attributes (Poivet et al., 2018; Renault, Coulon, de Revel, Barbe, & Bely, 2015; Sáenz-Navajas et al., 2016; Waterhouse, Sacks, & Jeffery, 2016). Even at a concentration below threshold, esters in alcohol solution can also impart aroma perception through synergistic effects (Lytra, Tempere, Le Floch, de Revel, & Barbe, 2013). The most important aromatic esters in wines include acetate esters and fatty acid ethyl esters, which are mainly produced by yeasts through their metabolism via the reaction between fatty acids and alcohols during alcoholic fermentation (Ebeler, 2001; Sumby, Grbin, & Jiranek, 2010). To enhance ester levels of wine mixed fermentation, various approaches have been considered, such as non-*Saccharomyces* strain selection (Domizio et al., 2011; Sáenz-Navajas et al., 2016), inoculation strategies (Hu, Jin, Xu, & Tao, 2018), and grape choice (Englezos et al., 2018). Although non-*Saccharomyces* strains will typically die off as the fermentation progresses, they act as the key contributor to ester enhancement by interacting with *S. cerevisiae*. During their growth interaction, nitrogen nutrient competition is thought to be one of the main drivers (Ciani & Comitini, 2015).

As a critical nutrient, yeast assimilable nitrogen (YAN) supports yeast growth and regulates sugar consumption during alcoholic fermentation. Grape must with a YAN concentration below 140–150 mg/L notably raises the risk of slow or stuck fermentation (Bell & Henschke, 2005; Butzke, 1998). To overcome nitrogen deficiency and assure an

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https://doi.org/10.1016/j.foodres.2019.05.030
Received 20 March 2019; Received in revised form 12 May 2019; Accepted 20 May 2019
Available online 21 May 2019

appropriate population of yeast, winemakers generally supplement YAN source, such as ammonium salts, or the mixture with amino acids, into grape musts (Bely, Sablayrolles, & Barre, 1990; Henschke & Jiranek, 1993; Torrea et al., 2011). Extensive studies on single fermentation with *S. cerevisiae* have revealed that YAN management is critical for shaping wine ester profiles. The increase in initial YAN content generally stimulates *S. cerevisiae* growth and acetate ester production; whereas fatty acid ethyl ester production shows uncertain relationship with initial YAN levels (Carrau et al., 2008; Saerens et al., 2008; Ugliano, Travis, Francis, & Henschke, 2010). This suggests that ethyl esters may be more related to fatty acid metabolism (Saerens et al., 2006). Moreover, when supplementing the same amount of YAN at different fermentation stages, *S. cerevisiae* biomass is not increased, whereas ester production is diversely influenced (Beltran, Esteve-Zarzoso, Rozès, Mas, & Guillamón, 2005; Seguinot et al., 2018). Therefore, nitrogen supplementation at different time-points during single *S. cerevisiae* fermentation seems to intervene in the existing pathway of ester production.

Since competition for nitrogen sources is a major driver of yeast succession during mixed culture fermentation, nitrogen supplementation may trigger greater influence on yeast behavior and thus can impact ester productivity. Regarding this attempt, only few efforts have been made, and mainly, these studies focus on yeast growth. For instance, Andorrà, Berradre, Mas, Esteve-Zarzoso, and Guillamón (2012) initially revealed that *S. cerevisiae* dominates over non-*Saccharomyces* yeast through highly taking up nitrogen sources. The non-*Saccharomyces* species could also affect nutrient availability for *S. cerevisiae* strain when inoculated prior to *S. cerevisiae* (Medina, Boido, Dellacassa, & Carrau, 2012). Following study indicated that the initial nitrogen concentration was not the factor affecting yeast growth interaction, but was able to impact ester components of final wines (Lage et al., 2014). Further work is, however, required to better understand the effects of nitrogen nutrient addition on ester production during mixed fermentation, which can enable the effective use of nitrogen nutrients, and the enhancement of ester profiles with lower winemaking costs.

We recently identified a *Hanseniaspora uvarum* Yun268 strain capable of enhancing wine ester content when fermented with *S. cerevisiae* F5. The final ester components were greatly affected by the growth interaction between two yeasts (Hu, Jin, Mei, Li, & Tao, 2018). In this work, mixed-culture fermentation of these two strains was conducted using a synthetic grape must to investigate yeast growth interaction and ester production dynamics under varying levels and timings of nitrogen addition. Furthermore, the levels of precursors and extracellular metabolites involved in ester production were determined throughout the fermentation, which can provide insights into the possible mechanism of ester production influenced by nitrogen nutrition addition.

2. Materials and methods

2.1. Chemical standards

Volatile chemical standards with purity ≥97.0% were from Sigma-Aldrich (Shanghai, China), including ethyl acetate, isobutyl acetate, isoamyl acetate, phenylethyl acetate, ethyl butyrate, ethyl isovalerate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, isobutyl alcohol, isoamyl alcohol, 2-phenylethanol, 2-octanol, hexanoic acid, octanoic acid, and decanoic acid (Table S1). Non-volatile standards (≥98.0% purity), i.e. citric acid, L-malic acid, acetic acid, succinic acid, and glycerol were from J&K (Beijing, China) (Table S2). Water was obtained from a Milli-Q purification system (Millipore, Bedford, USA).

2.2. Yeast strains and fermentation media

H. uvarum Yun268 was isolated from *Blue French* (*V. vinifera* L.) grape and identified using sequence analysis of the 26S rDNA D1/D2 domain (Hu et al., 2016). Commercial *S. cerevisiae* (Actiflore® F5) was purchased from the Laffort Wine Accessory Co., France. Prior to inoculation, yeast cells were cultivated at 28 °C with agitation at 170 rpm for 48 h in yeast-peptone-dextrose (YPD) medium (2% glucose, 2% peptone, 1% yeast extract, and unadjusted pH), collected by centrifugation at 4500 ×*g* for 3 min, and then washed twice with the 0.85% (*w/v*) NaCl solution.

To mimic winemaking practices and ensure reproducibility of media composition, a synthetic grape must that simulated natural grape must was used (Bely et al., 1990; Rollero et al., 2015). It contained 100 g/L glucose, 100 g/L fructose, 6 g/L malic acid, 6 g/L citric acid, 750 mg/L KH_2PO_4, 500 mg/L K_2SO_4, 250 mg/L $MgSO_4{\cdot}7H_2O$, 155 mg/L $CaCl_2{\cdot}2H_2O$, 200 mg/L NaCl, vitamins (20 mg/L myo-inositol, 1.5 mg/L calcium pantothenate, 0.223 mg/L thiamin hydrochloride, 2 mg/L nicotinic acid, 0.25 mg/L pyridoxine, and 0.003 mg/L biotin), and oligoelements (4 mg/L $MnSO_4{\cdot}H_2O$, 4 mg/L $ZnSO_4{\cdot}7H_2O$, 1 mg/L $CuSO_4{\cdot}5H_2O$, 0.4 mg/L $CoCl_2{\cdot}6H_2O$, 1 mg/L H_3BO_3, and 1 mg/L $(NH_4)_6Mo_7O_{24}$). The nitrogen sources were composed of ammonium chloride and amino acids. Their addition amounts could be changed to control YAN levels of the medium. The stock solution of amino acids included 1.4 g/L tyrosine, 13.7 g/L tryptophan, 2.5 g/L isoleucine, 3.4 g/L aspartate, 9.2 g/L glutamate, 28.6 g/L arginine, 3.7 g/L leucine, 5.8 g/L threonine, 1.4 g/L glycine, 38.6 g/L glutamine, 11.1 g/L alanine, 3.4 g/L valine, 2.4 g/L methionine, 2.9 g/L phenylalanine, 6.0 g/L serine, 2.5 g/L histidine, 1.3 g/L lysine, 1.0 g/L cysteine. The stock solution of amino acids also included 46.8 g/L proline, which, however, was not considered assimilable nitrogen (Beltran et al., 2005). YAN content was checked using the formaldehyde titration method (Zoecklein, Fugelsang, Gump, & Nury, 1995). Final pH value of the synthetic grape must was adjusted to 3.3 with 10 M sodium hydroxide.

2.3. Fermentation conditions

Static fermentations were conducted at 22 °C using 1 L blue cap flask (Shuniu®, Sichuan, China) containing 750 mL synthetic must in which 2×10^6 cells/mL of *H. uvarum* Yun268 and 4×10^6 cells/mL of *S. cerevisiae* F5 were simultaneously inoculated (Hu, Jin, Mei, et al., 2018). The flasks were sealed by hydrophobic membranes. During fermentation, CO_2 was released through an air outlet membrane. Yeast biomass was counted using Wallerstein nutrient (WL) agar medium that allowed *H. uvarum* Yun268 to grow as "green flat" colonies, and *S. cerevisiae* F5 as "white convex" colonies.

Regarding different levels of nitrogen addition, fermentation with threshold YAN content of 150 mg/L was the control (NL0). Treatments included supplementation of 50, 100, 150, and 200 mg/L YAN to NL0 at the point of yeast inoculation to obtain wines corresponding to NL50–NL200 (200–350 mg N/L). According to the result obtained from nitrogen addition levels, two treatments that performed similarly were used to evaluate whether nitrogen addition at various fermentation stages affected mixed fermentation significantly. Therefore, nitrogen additions of varying timings were performed by supplementing 50 mg/L YAN into the medium initially containing 250 mg/L YAN when the sugar content was 200 g/L (0 d, inoculation point, the control), 175 g/L (0.5 d), 150 g/L (1.5 d), 100 g/L (2 d), and 25 g/L (6 d), which corresponded to wines NT200 to NT25. Fermentations were considered complete when the sugar content dropped below 2 g/L. Wine samples were centrifuged at 4 °C and 4500 ×*g* for 5 min, filtered using a 0.22-μm membrane, and stored at −20 °C until further analysis. Each fermentation was done in duplicate.

2.4. Volatile analysis

Volatiles were analyzed using headspace solid-phase microextraction (HS-SPME) coupled with GC–MS adapted from Tao, Li, Wang, and Zhang (2008). A 50/30 μm DVB/CAR/PDMS fiber (Supelco, Bellefonte PA, USA) was used for volatile extraction. In a 20 mL gas-tight vial, 2 g NaCl, 2 mL wine, 6 mL pure water, and 20 μL internal standard (16 mg/

L, 2-octanol) were added, placed in a 40 °C water bath with stirring for 15 min at 600 rpm, extracted for 30 min, and then desorbed in the GC injection port (230 °C) for 5 min using a Shimadzu QP2020 GC–MS (Shimadzu Corporation, Kyoto, Japan) and a DB-WAX column (60 m × 0.25 mm × 0.25 μm, Agilent J & W, USA). The carrier gas was helium (99.999%) with flow rate of 1.5 mL/min. The GC program was as follows: 40 °C for 3 min, raised to 160 °C at 4 °C/min, followed by increase to 220 °C at 7 °C/min, and hold for 8 min. MS transfer line and ion source temperatures were 220 °C and 200 °C, respectively. Electron ionization (EI) mass spectrometric data from *m/z* 35 to 350 were scanned at 0.2 s intervals. Volatiles were identified by comparing their retention times and mass spectra with those of pure standards using the NIST 17 mass spectral library. Volatile concentrations were quantitated by interpolating the relative areas versus the area of the internal standard (2-octanol) using calibration graphs established for pure standards (Ferreira, López, & Cacho, 2000) (Table S1).

2.5. Non-volatile analysis

Citric acid, malic acid, acetic acid, and succinic acid were determined by a Shimadzu LC-2010A_{HT} HPLC (Shimadzu Corporation, Kyoto, Japan) with a Rezex™ ROA-Organic Acid H^+ (8%) column (150 × 7.8 mm; Phenomenex, USA) at 55 °C. The column was eluted with 2.5 mM H_2SO_4 at a flow rate of 0.6 mL/min. Compound concentrations were quantitated using the calibration curves built for pure standards (Table S2). Glycerol content was determined using a Y15 enzymatic autoanalyzer (Biosystems, Barcelona, Spain) with corresponding kit (http://www.biosystems.es).

2.6. Statistical analysis

All data were expressed as mean ± standard deviation. Total yeast biomass was quantitated as average biomass × survival time (day). Data differences were compared using one-way analysis of variance (ANOVA) with Duncan test, data correlation was measured using two-tailed Pearson correlation coefficient (*R*), and underlying relationship between fermentation characteristics and nitrogen addition were revealed using principal component analysis (PCA) of the SPSS 20.0 (SPSS Inc., Chicago IL, USA).

3. Results and discussion

All the fermentations were completed (residual sugars < 2 g/L), with approximately 11.0% *v*/v ethanol (Table S3). Yeast population dynamics and chemical composition remained the most similar profiles between wines of NL100 and NL150, corresponding to initial YAN levels of 250 mg/L and 300 mg/L. Thereby, 50 mg/L YAN was added into the medium initially containing 250 mg/L YAN at various fermentation stages to evaluate whether nitrogen addition with various timings affected mixed fermentation significantly.

3.1. Yeast growth in response to nitrogen nutrient additions

At different levels of nitrogen addition, yeast growth dynamics were slightly affected (Fig. 1a). Briefly, only at the addition level exceeding 200 mg N/L (NL200) the dominance of *S. cerevisiae* F5 over *H. uvarum* Yun268 was improved. Meanwhile, the increased biomass of *S. cerevisiae* F5 supported the positive effect of YAN content on the growth (Mendes-Ferreira et al., 2007; Tesnière, Brice, & Blondin, 2015). When supplementing nitrogen at different fermentation stages, the growth interaction between two yeasts was changed dramatically (Fig. 1b). For instance, nitrogen addition at the early stage (NT175) did not affect *H. uvarum* Yun268 growth compared to that at initial stage (NT200). As the addition delayed, however, the survival time of *H. uvarum* Yun268 was gradually increased by 1 day in NT150 and NT100, and by 2 days in NT25. The growth of *S. cerevisiae* F5 was simultaneously enhanced in NT100 and NT25, in which a higher maximum biomass was detected.

As reported previously, *S. cerevisiae* dominates over *H. uvarum* yeasts not only by producing killer metabolites (Wang, Mas, & Esteve-Zarzoso, 2015), but also by stronger competitiveness for nitrogen nutrients during co-fermentation (Andorrà et al., 2012). Our results suggested that nitrogen addition with high levels or early timings could drive the competition of *S. cerevisiae* F5 over *H. uvarum* Yun268. However, their competition could be eased when nitrogen addition was introduced at middle or later stages, which may result into certain response of ester production.

3.2. Ester production under nitrogen nutrient additions

3.2.1. Acetates of higher alcohols

Yeasts catabolize nitrogen source to form higher alcohols via the Ehrlich pathway (Swiegers, Bartowsky, Henschke, & Pretorius, 2005), thereby directly defining the production of resulting acetate esters, namely acetates of higher alcohols (AHAs). Three kinds of AHAs were characterized in final synthetic wines, including isobutyl acetate, isoamyl acetate, and phenethyl acetate (Table 1). Under different levels of nitrogen addition, the concentrations of the AHAs seemed to be unchanged. Interestingly, a striking increment of their concentrations was observed in NT100, with 1.6-fold isobutyl acetate, 3.1-fold isoamyl acetate, and 1.4-fold phenethyl acetate higher than that in NT200. Nitrogen addition in the late stage enhanced these acetate esters to a lesser extent in NT25, while early-stage additions in NT150 and NT175 affected AHA profiles negligibly. We have characterized *H. uvarum* Yun268 as a strong producer of AHAs when co-fermented with *S. cerevisiae* species (Hu, Jin, Mei, et al., 2018; Hu, Jin, Xu, & Tao, 2018). Thus, the increase of AHA levels in NT100 and NT25 fitted with the increased *H. uvarum* Yun268 biomass. This positive relationship was clearly found in the variation of ethyl acetate, a metabolic marker of *Hanseniaspora* species (Domizio et al., 2011).

To provide insight into the addition process, the production dynamics of these AHAs in three typical conditions (i.e. NT200, NT100 and NT25) were analyzed (Fig. 2a, for details see Fig. S1a, S1b, and S1c). We detected a rapid increase of total AHA production when nitrogen was supplemented in NT200. Such positive response was more pronounced in NT100, of which the maximum concentration of AHAs was 1.2-fold higher. Nitrogen addition in NT25 had minimal effect on the maximum concentration (especially for phenethyl acetate, Fig. S1c), but prevented their sharp decline at later stages. The data proved that nitrogen addition at the middle stage increased the AHA production during mixed fermentation. Notably, the total concentrations of AHAs in NT100 and NT25 exceeded that in NT200 occurred at days 4 and 8, respectively. At the both time points *H. uvarum* Yun268 yeasts had been replaced by *S. cerevisiae* F5 (Fig. 1b), indicating that *H. uvarum*-induced increase in acetate ester levels occurred during the death phase of *H. uvarum* Yun268 and not during their growth stage. This observation was quite similar with the impact of *Lachancea thermotolerans* on chemical changes of mixed fermentation (Peng, Viana, Petersen, Larsen, & Arneborg, 2018).

3.2.2. Fatty acid ethyl esters

Fatty acid ethyl esters are mainly produced from fatty acids involving in yeast carbon nutrition (Swiegers et al., 2005). We thus evaluated whether the nitrogen addition enabled the enhancement of these esters by affecting central carbon pathway. Despite no response from short-chain fatty acid levels, medium-chain fatty acid (MCFA) ethyl esters, such as ethyl octanoate and ethyl decanoate, were found as the targeted components induced by nitrogen addition (Table 1). Similarly, NT100 significantly increased their final concentrations in comparison with NT200. Such gap increased from 0%, 10% to 39% as the carbon chain length increased from C6, C8 to C10, which implied that fatty acid chain extension may be also induced by the nitrogen addition. This issue, however, needs to be further investigated. Different levels of

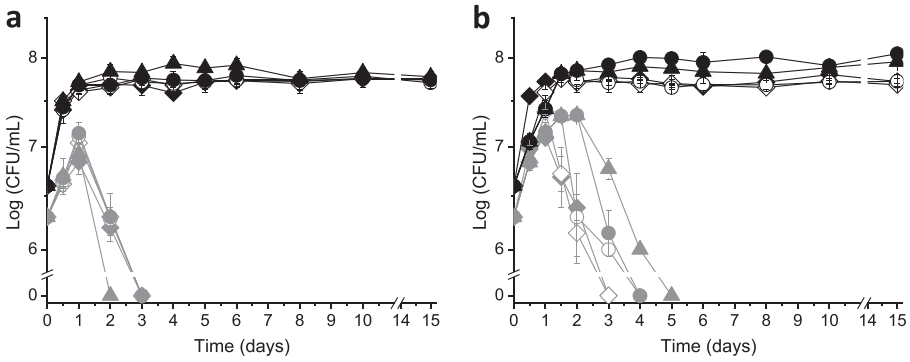

Fig. 1. Yeast populations of *H. uvarum* Yun268 (gray line) and *S. cerevisiae* F5 (black line) in mixed fermentations. (a) Nitrogen addition levels: NL0 (solid diamond), NL50 (open diamond), NL100 (open circle), NL150 (solid circle), and NL200 (solid triangle). (b) Nitrogen addition timings: NT200 (solid diamond), NT175 (open diamond), NT150 (open circle), NT100 (solid circle), and NT25 (solid triangle). Data are mean ± standard deviation.

nitrogen addition only increased ethyl decanoate concentration at addition level beyond 200 mg/L (NL200). Whereas, supplementing grape must with 200 mg/L YAN exceeded the enological limits recommended in Europe (64 mg N/L) and Australia (85 mg N/L) (Fugelsang & Edwards, 2007).

Production dynamics of ethyl octanoate and ethyl decanoate further proved the highest productivity triggered in NT100 (Fig. 2b, for details see Fig. S1d and e). Similar to the modulation of AHAs, nitrogen addition caused a quick inducement of MCFA ethyl ester production, and their concentrations in NT100 and NT25 exceeded that in NT200 occurred during the death phase of *H. uvarum* Yun268. However, both MCFA ethyl esters and acetate esters in all fermentations decreased at later stages, which may be attributed to the increase in the hydrolysis-related esterase activity (Mauricio et al., 1993). Previously, we have shown that the raise in MCFA ethyl ester levels during mixed fermentation is a biomass-dependent process, in which *H. uvarum* Yun268 contributed to the increase of the precursor (fatty acids) level, and *S. cerevisiae* F5 provided high esterase activity (Hu, Jin, Mei, et al., 2018). Little response of MCFA ethyl ester production at different levels of nitrogen addition was consistent with the slight changes of yeast growth. Indeed, the greatest increase of MCFA ethyl ester production was achieved in NT100 where the growth of both *H. uvarum* Yun268 and *S. cerevisiae* F5 increased.

The above results suggested that the timings but not the levels of nitrogen addition markedly encouraged the ester production during mixed fermentation. Nitrogen addition at the middle stage gained the greatest outcome, which seemed to associate with the eased competition between two yeasts.

3.3. Possible mechanism for ester enhancement under nitrogen nutrient additions

To illustrate the chemical mechanism by which nitrogen additions enhanced ester productions, the corresponding ester precursors were assessed. For the final concentrations, both higher alcohols and fatty acids showed a clear relationship with the timings than the levels of nitrogen addition (Table 1). Compared with NT200, the early-stage addition in NT175 and NT150 hardly changed the profiles of higher alcohols and MCFAs, such as isobutyl/isoamyl/2-phenyl alcohols and octanoic/decanoic acids. Surprisingly, their concentrations were dramatically increased as the addition performed in NT100 and NT25. Production dynamics further revealed that these higher alcohols were supremely enhanced in NT100 but were suppressed in NT200 (Fig. 2c, for details see Fig. S2a, S2b, and S2c). In contrast, the production dynamics of MCFA precursors were well fitted with that of MCFA ethyl esters (Fig. 2d, for details see Fig. S2d and e), and the overall decrease in MCFA production during the later stage may derive from weak carbon metabolism activity of yeast (Swiegers et al., 2005). As a result, the Pearson correlation coefficient between higher alcohol and AHA production was rather low ($R = 0.347$), while R value between MCFA and MCFA ethyl ester production reached 0.744 (Fig. 2e and f). The result supported that the fatty acid level is the most limiting factor in the ethyl ester production (Saerens et al., 2006), and thus the increase of MCFA ethyl ester production may possibly benefit from the enhanced MCFA metabolism that was induced by nitrogen addition.

To prove our hypothesis, yeast extracellular metabolites involved in MCFA pathway were analyzed. It was found that NL100 and NL150 had the similar metabolite profiles, such as glycerol and organic acids (Table S4). Surprisingly, supplementing 50 mg/L YAN at the middle stage (NT100) dramatically influenced the existing pathway, resulting into a decreased production of glycerol and acetic acid, but an increased production of succinic acid during fermentation (Fig. 3a, b and c). Such changes indicated that the carbon fluxes tended to participate in the formation of pyruvate and acetyl CoA, the precursors for the MCFA production. A previous study reported a similar positive effect of nitrogen management on the fatty acid biosynthesis, with intensified glycolysis, impaired TCA cycle and enhanced metabolic fluxes channeling pyruvate and acetyl-CoA to fatty acids (Zhu et al., 2012). In this study, the enhanced MCFA production was achieved in NT100 owing to the suppressed production of glycerol and acetic acid. Taken together, nitrogen addition at the middle stage seemed to trigger a redistribution of carbon fluxes in the central pathway, which finally activated the production of MCFAs and their ethyl esters (Fig. 4).

Ester compositions, total yeast biomass, precursor levels, and extracellular metabolites under different timings of nitrogen addition were analyzed through PCA to further explore their underlying relationships (Fig. 5). Both *H. uvarum* Yun268 and *S. cerevisiae* F5 were located closely to NT100 and NT25 at the positive part of PC1 (76.8%) and PC2 (17.8%). The enhanced growth of two yeasts may take responsible for the greatest outcomes of esters and precursors in these two treatments, and especially in NT100. Succinic acid was positively related to both MCFAs and their ethyl esters, as opposed to acetic acid and glycerol. Therefore, the increase in succinic acid level could be considered as a metabolic indicator of the enhanced MCFA ethyl ester production that was induced by the nitrogen addition. NT175 and NT150, however, clustered together with NT200 which stayed away from esters, emphasizing the poor efficiency of nitrogen additions at the early stage of mixed fermentation.

4. Conclusion

In conclusion, this work provides an insight into the ester production in response to nitrogen nutrient management during mixed fermentation of a synthetic grape must. Our results demonstrated that the timings rather than the levels of nitrogen addition drove ester productions during mixed fermentation with *H. uvarum* Yun268 and *S. cerevisiae* F5. Nitrogen addition at the early stage increased the dominance of *S. cerevisiae* over *H. uvarum* Yun268, which reduced the contribution of *H. uvarum* Yun268 to ester production. Supplementing YAN at the mid-stage significantly eased yeast competition. As a result, the increased survival of *H. uvarum* Yun268 contributed to the production of higher alcohol acetates, such as isobutyl acetate, isoamyl

Table 1
Concentrations of esters and their precursors in the synthetic wines obtained from different levels and timings of nitrogen addition.

Compounds	Nitrogen addition level					Nitrogen addition timing				
	NL0	NL50	NL100	NL150	NL200	NT200	NT175	NT150	NT100	NT25
Ethyl acetate	**31,685 ± 1899^{b}**	**33,403 ± 1202^{b}**	**34,983 ± 2036^{b}**	**32,092 ± 4808^{b}**	23,605 ± 1929^{a}	29,254 ± 2321^{A}	29,730 ± 1411^{A}	31,813 ± 2333^{AB}	35,491 ± 1048^{BC}	**39,491 ± 1414^{C}**
Acetates of higher alcohols (AHAs) (μg/L)										
Isobutyl acetate	19 ± 1	18 ± 3	20 ± 1	19 ± 1	20 ± 2	18 ± 2^{A}	16 ± 3^{A}	19 ± 1^{A}	**28 ± 4^{B}**	20 ± 1^{A}
Isoamyl acetate	160 ± 7	163 ± 27	164 ± 17	140 ± 31	172 ± 25	189 ± 24^{A}	213 ± 7^{A}	231 ± 10^{A}	**586 ± 31^{C}**	418 ± 15^{B}
Phenethyl acetate	288 ± 12	275 ± 29	250 ± 1	255 ± 24	297 ± 46	253 ± 10^{B}	233 ± 8^{AB}	211 ± 13^{A}	**364 ± 8^{C}**	229 ± 23^{AB}
Σ	467 ± 20	456 ± 59	434 ± 19	414 ± 56	489 ± 73	460 ± 36^{A}	462 ± 18^{A}	461 ± 24^{A}	978 ± 43^{C}	667 ± 39^{B}
Short-chain fatty acid ethyl esters (μg/L)										
Ethyl butyrate	144 ± 16	165 ± 4	152 ± 16	150 ± 1	166 ± 11	113 ± 6	133 ± 25	118 ± 8	134 ± 20	132 ± 10
Ethyl isovalerate	3 ± 0	3 ± 0	3 ± 1	3 ± 0	3 ± 0	2 ± 0	2 ± 0	2 ± 1	3 ± 1	2 ± 0
Σ	147 ± 16	168 ± 4	155 ± 17	153 ± 1	169 ± 11	115 ± 6	135 ± 25	120 ± 9	137 ± 21	134 ± 10
Medium-chain fatty acid ethyl esters (MCFAEEs) (μg/L)										
Ethyl hexanoate	547 ± 39	663 ± 61	597 ± 6	593 ± 29	612 ± 5	504 ± 32	522 ± 30	512 ± 34	502 ± 40	502 ± 41
Ethyl octanoate	447 ± 51	512 ± 57	483 ± 31	435 ± 37	482 ± 21	473 ± 30^{AB}	467 ± 23^{AB}	485 ± 20^{AB}	**519 ± 15^{B}**	439 ± 35^{A}
Ethyl decanoate	54 ± 8^{ab}	62 ± 7^{ab}	52 ± 3^{ab}	48 ± 6^{a}	**68 ± 3^{b}**	44 ± 4^{AB}	45 ± 4^{AB}	40 ± 4^{A}	**61 ± 3^{C}**	55 ± 8^{BC}
Σ	1048 ± 98	1237 ± 125	1132 ± 94	1076 ± 72	1162 ± 29	1021 ± 66	1034 ± 57	1037 ± 58	1082 ± 58	996 ± 84
Higher alcohols (HAs) (mg/L)										
Isobutyl alcohol	35.81 ± 1.86	30.53 ± 4.80	34.53 ± 1.55	30.03 ± 3.68	38.19 ± 4.32	30.46 ± 3.55^{A}	30.51 ± 2.84^{A}	29.40 ± 4.22^{A}	**53.00 ± 9.58^{B}**	**46.60 ± 8.48^{B}**
Isoamyl alcohol	77.24 ± 4.49	78.51 ± 3.34	78.99 ± 3.45	79.74 ± 5.54	85.10 ± 4.71	84.94 ± 7.45^{A}	83.66 ± 3.51^{A}	81.72 ± 4.81^{A}	**134.70 ± 12.36^{B}**	**122.22 ± 9.81^{B}**
2-Phenylethanol	**65.09 ± 4.65^{c}**	48.45 ± 2.91^{ab}	43.71 ± 0.31^{a}	47.17 ± 1.20^{ab}	53.47 ± 3.61^{b}	42.69 ± 2.47^{A}	45.70 ± 3.07^{A}	44.98 ± 6.55^{A}	**95.20 ± 1.82^{C}**	70.82 ± 2.28^{B}
Σ	178.14 ± 11.00	157.49 ± 11.05	157.23 ± 5.31	156.94 ± 10.42	176.76 ± 12.64	158.09 ± 13.47^{A}	159.87 ± 9.42^{A}	156.10 ± 15.58^{A}	282.90 ± 23.76^{B}	239.64 ± 20.57^{B}
Medium-chain fatty acids (MCFAs) (mg/L)										
Hexanoic acid	5.18 ± 0.59^{ab}	**5.83 ± 0.29^{b}**	5.12 ± 0.71^{ab}	4.89 ± 0.32^{ab}	4.40 ± 0.09^{a}	4.06 ± 0.17	4.33 ± 0.22	4.06 ± 0.31	3.84 ± 0.32	3.65 ± 0.29
Octanoic acid	**5.97 ± 0.30^{b}**	**5.59 ± 0.38^{b}**	4.25 ± 0.18^{a}	4.98 ± 0.18^{ab}	**5.47 ± 0.78^{b}**	4.11 ± 0.29^{A}	4.30 ± 0.22^{AB}	4.12 ± 0.34^{A}	**5.09 ± 0.16^{B}**	4.43 ± 0.41^{AB}
Decanoic acid	**1.11 ± 0.01^{d}**	0.79 ± 0.10^{bc}	0.53 ± 0.07^{a}	0.70 ± 0.03^{b}	0.88 ± 0.05^{c}	0.45 ± 0.01^{AB}	0.52 ± 0.03^{AB}	0.44 ± 0.03^{A}	0.54 ± 0.05^{B}	**0.64 ± 0.04^{C}**
Σ	**12.26 ± 0.90^{b}**	**12.21 ± 0.77^{b}**	9.90 ± 0.96^{a}	10.57 ± 0.53^{ab}	10.75 ± 0.92^{ab}	8.62 ± 0.47	9.15 ± 0.47	8.62 ± 0.68	9.47 ± 0.53	8.72 ± 0.74

Data are mean ± standard deviation. Values displaying different letters within each row are significantly different according to the Duncan test at 95% confidence level. The highest concentration was presented in bold font.

NL0, NL50, NL100, NL150, and NL200: wines obtained from different levels of nitrogen addition through supplementing 0, 50, 100, 150, and 200 mg/L YAN into the medium initially containing 150 mg/L YAN, respectively.

NT200, NT175, NT150, NT100, and NT25: wines obtained from different timings of nitrogen addition when the sugar content was 200, 175, 150, 100, and 25 g/L, respectively.

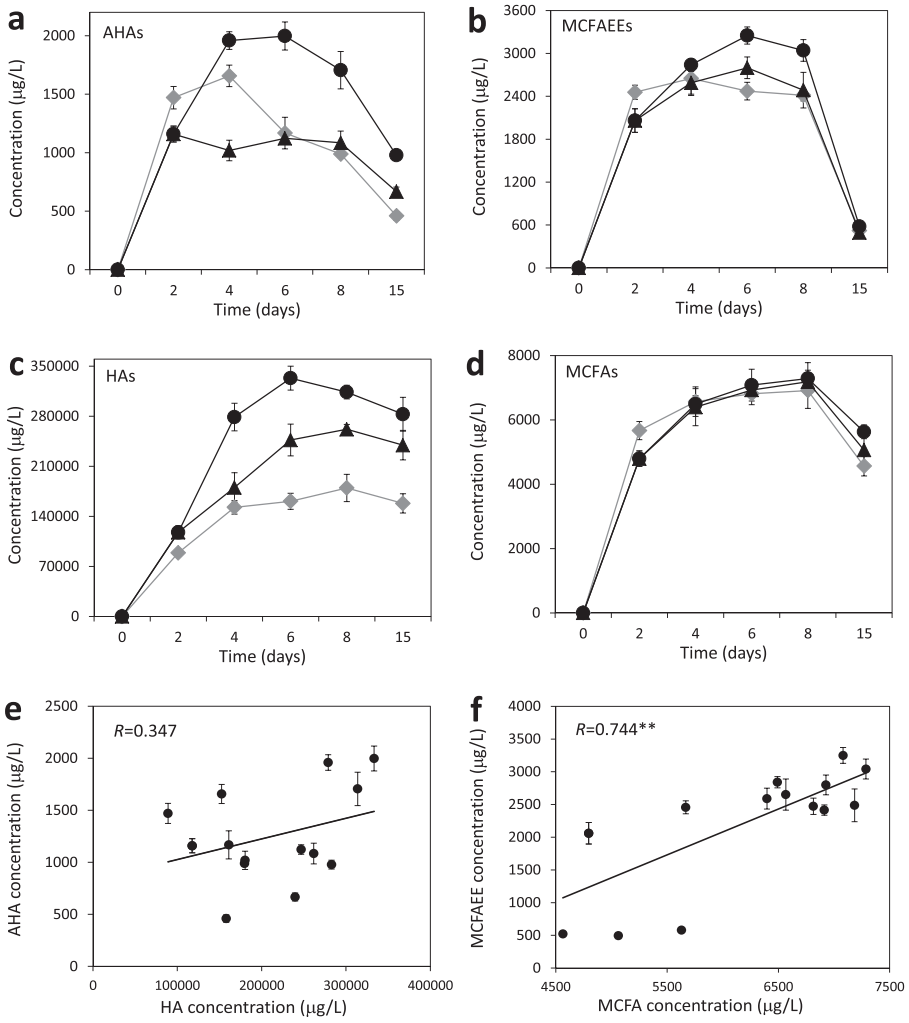

Fig. 2. Production dynamics of esters and their precursors under three typical timings of nitrogen addition: NT200 (◆), NT100 (●), and NT25 (▲). (a) AHAs, acetates of higher alcohols = Σ(isobutyl acetate + isoamyl acetate + phenethyl acetate); (b) MCFAEEs, medium-chain fatty acid ethyl esters = Σ(ethyl octanoate + ethyl decanoate); (c) HAs, higher alcohols = Σ (isobutyl alcohol + isoamyl alcohol +2-phenylethanol); (d) MCFAs, medium-chain fatty acids = Σ(octanoic acid + decanoic acid); (e) Pearson correlation scatter plot of AHA and HA production; (f) Pearson correlation scatter plot of MCFAEE and MCFA production. Correlation is significant at 99% (**) confidence level. Data are mean ± standard deviation.

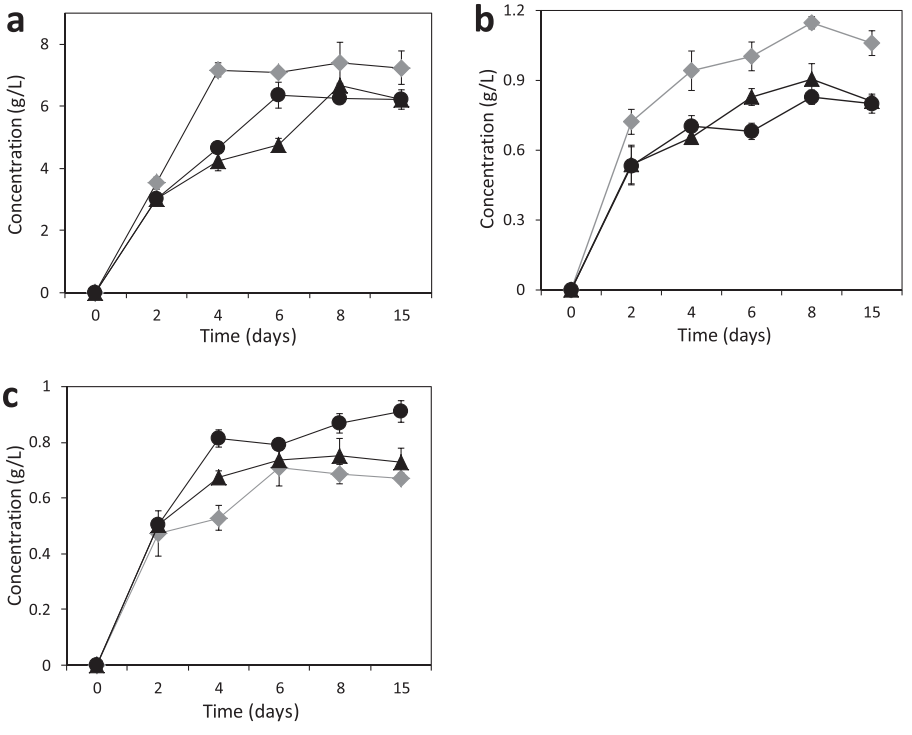

Fig. 3. The accumulation of (a) glycerol, (b) acetic acid, and (c) succinic acid under three typical timings of nitrogen addition: NT200 (◆), NT100 (●), and NT25 (▲). Data are mean ± standard deviation.

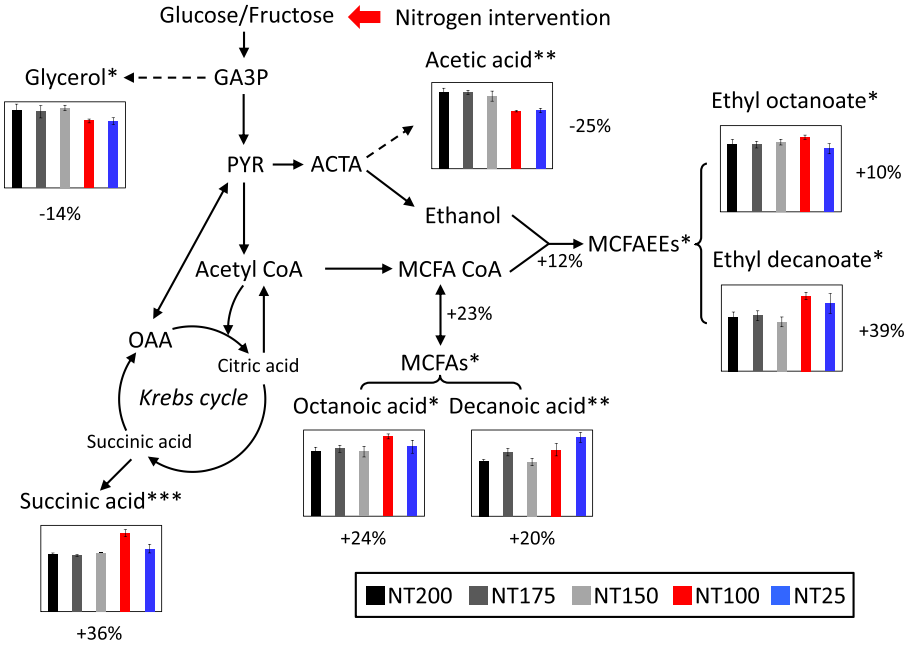

Fig. 4. The redistribution of carbon fluxes in the central metabolism network under different timings of nitrogen addition. PYR, pyruvate; GA3P, Glyceraldehyde 3-phosphate; ACTA, acetaldehyde; MCFAEEs, medium-chain fatty acid ethyl esters; MCFAs, medium-chain fatty acids. Data are mean ± standard deviation. Difference significant at 95% (*), 99% (**), and 99.9% (***) confidence level. Changes of metabolite levels in NT100 are given as a percentage compared to that in NT200.

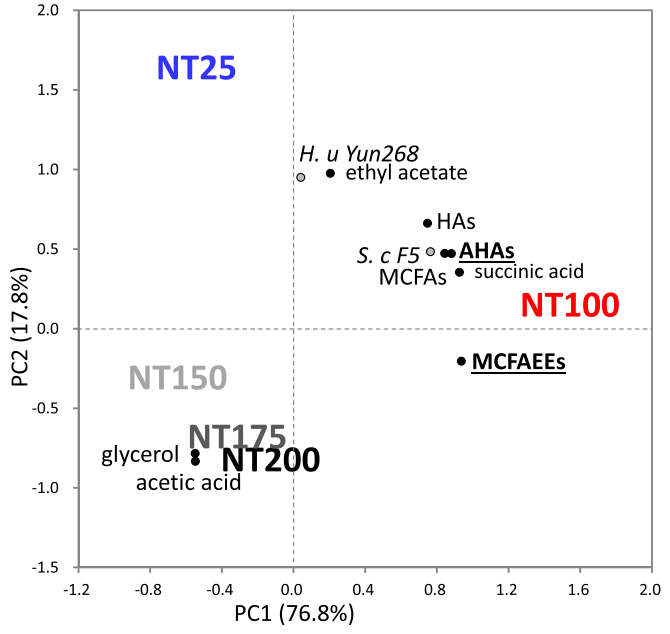

Fig. 5. PCA of fermentation characteristics obtained from different timings of nitrogen addition. AHAs, acetates of higher alcohols; HAs, higher alcohols; MCFAEEs, medium-chain fatty acid ethyl esters; MCFAs, medium-chain fatty acids. *H. uvarum* Yun268 and *S. cerevisiae* F5 was quantitated as total biomass.

acetate and phenethyl acetate. Meanwhile, the MCFA metabolism was promoted may derive from a redistribution of carbon fluxes in the central pathway, which enhanced corresponding MCFA ethyl ester production. Since fermentation performance or aroma production can be also affected by nitrogen sources (Kemsawasd, Viana, Ardö, & Arneborg, 2015; Liu, Yu, Li, Duan, & Yan, 2018), further work is needed to elucidate the effect of nitrogen sources on ester biosynthesis of these two yeasts during mixed fermentation, and to understand the sensory changes of final wines by using natural grape must.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (31771966 and 31801528), the Science and Technology Project of China Central Finance (106001000000150012), and State Scholarship Fund of China Scholarship Council (201806300074 and 201806305042).

Conflict of interest

The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2019.05.030.

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