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The Effect of Non-Saccharomyces Cerevisiae *Torulaspora delbrueckii* on the Aroma Composition of Munage Grape Base-Wine and the Mechanism of the Effect

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Abstract: To enhance comprehension of the impact of mixed fermentation using *Torulaspora delbrueckii* Bio-119667 (TD) on the aroma composition of Munage grape base-wine (MGBW), we analysed the aroma composition of MGBW using HS-SPME-GC-MS widely targeted metabolomics. The levels of volatile aroma components, including terpenes, higher alcohols, aldehydes, heterocyclic compounds, and esters, were significantly higher in MGBW produced by mixed fermentation compared to the pure *Saccharomyces* yeast control fermentation. The study found that the content of esters increased by 26.3% after mixed fermentation, indicating the contribution of TD to the formation of ester flavour components during the fermentation of MGBW. After analysing aroma activity values, we discovered that 49 out of 115 esters (25.5% of the total) significantly contributed to the aroma profile of MGBW (rOAV > 1). Of these esters, 16 were identified as key aroma compounds (rOAV > 1, VIP > 1) produced by mixed fermentation with the participation of TD. This finding further supports the contribution of TD to the improvement of MGBW's aroma composition. This study reveals the role of non-Saccharomyces yeast strain *Torulaspora delbrueckii* Bio-119667 in improving the aroma composition of MGBW produced by mixed culture fermentation and the biosynthetic pathways of key aroma components therein.

Keywords: Munage wine; mixed fermentation; metabolomics; HS-SPME-GC-MS; aroma composition; biosynthetic pathways

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1. Introduction

Munage grapes (*Vitis vinifera* L. cv. *Munage*) are a high-quality variety selected from local strains by the Agricultural Development Centre of Xinjiang Kizilsu Kyrgyz Autonomous Prefecture [1]. They are popular among consumers due to their large fruit size, sweet taste, and firm flesh. However, table grapes are vulnerable to fungi and pathogenic bacteria during post-harvest storage due to their thin skin. This vulnerability can result in water loss, browning, stem breakage, fruit loss, and even rotting and deterioration [2]. Post-harvest losses of table grapes account for 25% and 50% of total production in developed and developing countries, respectively [3]. Therefore, researchers in the field of freshness and post-harvest storage are conducting extensive research to extend the storage period and enhance the quality of Munage grapes. Teles et al. demonstrated that the use of high CO_2 in combination with atmospheric storage is a commercially viable option for controlling grey mould on organic 'Flame Bleu' and 'Crimson Bleu' table grapes [4]. Li et al. found that the endophytic fungus *Albifimbria verrucaria-SYE-1* inhibits the growth of grey mould on grapes and has broad biocontrol activity, making it a potential biocontrol agent for grapevine grey

mould with a wide range of biocontrol activities [5]. Furthermore, researching the food processing of Munage grapes offers an alternative solution to this issue. Grapes have a range of processed products, including grape juice, sultanas, grape vinegar, and grape jam. However, one of the most valuable, popular, and nutritious products is wine. The dynamic changes that occur during the processing of grapes into value-added products such as juices, jams, and vinegars are due to a variety of technological factors affecting the biologically active components [6–8].

The involvement of non-Saccharomyces in wine processing can produce a range of complex flavour compounds, including esters, alcohols, and acids, and can enhance the metabolic performance of *Saccharomyces*. Lencioni et al. used *Z. horentina* and *Starmerella bacillaris* to ferment high-sugar grape musts with *S. cerevisiae* which could effectively reduce the content of volatile acids in the finished wines [9]. Lu et al. utilised a blend of TD and *P. kluyveri* to ferment a grenadine wine, which was able to increase the aromatic complexity of the wine [10]. Zhang et al. used screened wild non-Saccharomyces TD and *S. cerevisiae* in a laboratory-scale mixed fermentation to produce red wine, which was found to increase the diversity of the wine's aroma and improve the quality of the product [11]. Englezos et al. utilised commercial *S. bacillaris* with *S. cerevisiae* to ferment four white wines, Chardonnay, Muscat, Riesling, and Sauvignon blanc, and found that this blended fermentation had a great potential to influence and modulate the chemical and aromatic characteristics of white wines, especially the white wines produced from Sauvignon blanc grapes [12]. Zhang et al. used *H. vineae* and *M. pulcherrima* to ferment ice wines with a blend of *S. cerevisiae*, and the results showed that *H. vineae* had unexpectedly good winemaking properties and that mixed fermentation could improve the aromatic diversity of ice wines [13].

The use of mixed fermentation with non-Saccharomyces yeasts and *Saccharomyces* yeasts not only increases the diversity of strains in the fermentation but also improves the flavour of wine, gives it a multilayered structure, and enhances the complexity of aroma and flavour. This presents a new opportunity for the winemaking industry [14]. Research has demonstrated that mixed fermentation, utilising multiple strains, can be adjusted to produce the desired metabolites for winemakers. This can result in an increase in the wine's aroma and complexity [15]. Howell et al. demonstrated that mixed fermentation of two strains can result in a distinctive composition of aroma substances, possibly due to a shared metabolic intermediate resulting from interactions between the two yeast strains. Additionally, they discovered that the aroma components in wines produced by mixed fermentation of two strains were not merely a proportional mixture of aroma components produced by the two strains alone [16].

Torulaspora delbrueckii (TD) is a common microorganism found in vineyards, grape skins, and winemaking environments [17]. Its cells are small, subcircular (6.5 μm × 5.5 μm), and the colonies on WLN culture medium are cream-coloured with a faint greenish tinge, spherical protrusions, and are smooth and opaque. It is a typical representative of the natural microbiota found on the surface of grapes. Its main feature is that it can slowly ferment a large amount of sugar, with an alcohol production capacity of 8% to 14%, and its fermentation products contain less acetic acid, ethyl acetate, and acetaldehyde [18]. Compared to *Saccharomyces cerevisiae*, TD produces less ethanol, making it ideal for the production of low-alcohol wines [19]. Studies have shown that it also produces lower levels of ethyl acetate, acetic acid, and acetaldehyde, and has a slower fermentation process [20]. As well as producing lower levels of ethyl acetate, acetic acid, and acetaldehyde, it also slowly ferments large amounts of sugar to produce more glycerol [21]. TD can affect the aroma of wine by affecting some chemical groups (e.g., increase in 2-phenylethanol). It also affects the production of volatile lipid substances, such as a decrease in isoamyl acetate C_6–C_{10} fatty acid content. It has been shown that the odor activity of lipid fatty acids is high in wines fermented with a mixture of TD and *Saccharomyces cerevisiae* and that TD can affect the aroma of wines by influencing carbonyl compounds [22,23]. This has a positive effect on the organoleptic properties of wines. Additionally, TD has good environmental tolerance, particularly high sugar tolerance. TD was isolated from high-sugar grape musts and

was found to be hyperosmotic-tolerant, and TD remained characterised by high glycerol production and low volatile acid production relative to *Saccharomyces cerevisiae*. Therefore, TD can be a potential strain for reducing volatile acids in wine fermentation, and the mixed fermentation of TD and *Saccharomyces cerevisiae* produces dry white wines and sweet wines with outstanding quality [24,25]. Therefore, the high adaptability, aroma enhancement, and alcohol reduction properties of TD make it a more favourable option for the development of low-alcohol Munage-grape-based wines.

Therefore, we analysed the aroma composition of Munage grape base-wine (MGBW) using HS-SPME-GC-MS broadly targeted metabolomics. We determined the improvement of the aroma composition of Munage-grape-based wines by using a mixed fermentation of TD with *Saccharomyces cerevisiae*. Additionally, we searched for key aroma compounds and their biosynthetic pathways in the aroma composition. This study aims to enhance the understanding of the role of TD in improving the aroma composition of MGBW. The results of this study will provide a new means for flavour enhancement of MGBW products.

2. Materials and Methods

2.1. Materials and Chemicals

For this study, Munage grapes (*Vitis vinifera* L. cv. *Munage*) used in this study were harvested from vineyards in Atushi City, Kizilsu-Kirghiz Autonomous Prefecture, Xinjiang, China, and had a fruit sugar content (as glucose) of 305.263 g/L, an acidity content (as tartaric acid) of 3.274 g/L, and a pH value of 4.64. Prior to winemaking, the grapes were stored at −20 °C in a dark environment.

Saccharomyces cerevisiae EC-1118 (EC-1118) and *Torulaspora delbrueckii* Bio-119667 (TD) were purchased in lyophilised form (Beijing Baio Bowei Biotechnology Co., Ltd., Beijing, China).

All chemical reagents used in this study, NaCl and n-hexane, were of chromatographic grade and were purchased from Kehua Weiye Reagent Distribution Department (Urumqi, Xinjiang, China).

2.2. The Making of MGBW

After thawing, Munage grapes (*Vitis vinifera* L. cv. *Munage*) were washed, de-stemmed, and lightly crushed. They were then pasteurised by heating them to 60 °C for 40 min. After the grape juice cooled, it was filtered through sterile gauze on an ultra-clean bench. Subsequently, 600 mL of grape juice was transferred to a sterile 1000 mL glass bottle. The juice's pH was adjusted to 3.61 by adding a 0.2 M citrate aqueous solution drop by drop, while monitoring it with a pH meter. The grape juice was inoculated with *Saccharomyces cerevisiae* EC-1118 (10^8 CFU/mL, 8 mL) and *Torulaspora delbrueckii* Bio-119667 (10^8 CFU/mL, 16 mL) and incubated at 28 °C for 96 h. After fermentation, the wine was clarified by centrifugation at 4000 r/min for 20 min. Please refer to Figure 1 for a visual representation of the process.

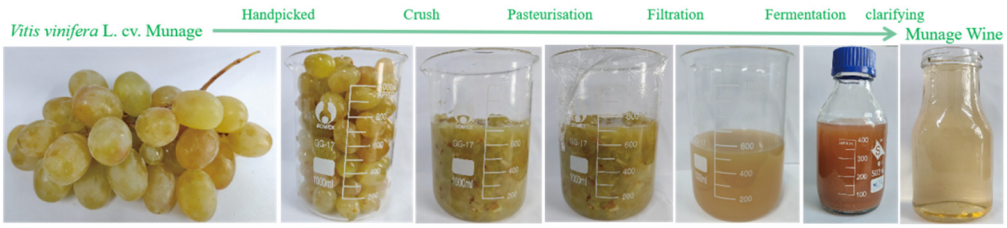

Figure 1. Schematic diagram of Munage Grape Base-Wine fermentation process.

2.3. Analysis of Physical and Chemical Properties

MGBW was analysed for pH, total acid (TA), total soluble solids (TS), and alcohol content. pH was determined using a bench-top digital pH meter. TA content was determined by potentiometric titration with an endpoint of pH 8.2. TS were determined by direct titration. Alcohol content was determined using an alcohol meter (M277465, Beijing,

China). All treatments were repeated three times in the laboratory and the results were expressed as the mean ± standard deviation of the three treatments.

2.4. Widely Targeted Metabolomics Analysis

2.4.1. Sample Preparation and Treatment

Sampling was performed at 0, 48, 72, and 96 h during fermentation and the single bacterial fermentation (*Saccharomyces cerevisiae* EC-1118, *Torulaspora delbrueckii* Bio-119667) broth was used as a control for the same time period, noted as Mix, EC1118, and TD, respectively.

First, 1 mL of sample was added to a 20 mL headspace bottle (Agilent, Santa Clara, CA, USA) containing saturated NaCl solution. Then, the headspace vial was sealed using a TFE silicone headspace septum rolled edge cap. For SPME analysis, each vial was equalised at 60 °C for 5 min. Subsequently, a 120 μm DVB/CWR/PDMS fibre was brought into contact with the sample headspace and left for 15 min at 60 °C.

2.4.2. GC-MS Analysis

Following the extraction process, the fibre coatings' volatile organic compounds (VOCs) were desorbed at 250 °C for 5 min in splitless mode at the inlet of an Agilent 8890 gas chromatograph. Qualitative and quantitative analyses of the volatile metabolites were performed using GC-MS (Agilent, 8890 and 7000D). A DB-5MS (30 m × 0.25 mm × 0.25 μm) capillary column was used. The carrier gas was helium at a flow rate of 1.2 mL/min. The inlet temperature is 250 °C. The ramp-up programme was set to increase the temperature from 40 °C to 100 °C at 10 °C/min, then to 180 °C at 7 °C/min, and finally to 280 °C at 25 °C/min for 5 min. The mass spectrum was recorded in 70 eV EI ionisation mode. The transmission line, ion source, and quadrupole mass detector were set to 230 °C, 280 °C, and 150 °C. The mass spectrometer operated in SIM mode for identification and quantification.

2.4.3. Metabolite rOAV Analysis

Relative odour activity value (rOAV) is a method of elucidating the contribution of each aroma compound to the overall aroma profile of a sample in conjunction with the sensory threshold of the compound. In general, when rOAV $\geq$ 1, it indicates that the compound directly contributes to the sample's flavour. The formula for calculating this is as follows:

$$rOAV_i = \frac{C_i}{T_i}$$

where $rOAV_i$ is the relative odor activity value of compound i; C_i is the relative content of the compound (μg/g or μg/mL); T_i is the threshold value of the compound (threshold, μg/g or μg/mL).

2.5. Pathway Verification

Gas chromatography was used to observe changes in α-linolenic acid content in the fermentation broths of mixed fermentation at 0 and 96 h in order to confirm the existence of α-linolenic acid metabolism during mixed fermentation. The GC separation of the samples was performed using a DB-WAX chromatographic column (60 m × 0.32 mm × 0.50 μm, 7-inch column) and an Agilent Technologies Inc. 6890 instrument. The initial column temperature was 160 °C. The temperature increase programme was set to warm up from 160 °C to 220 °C at 1.8 °C/min for 5 min. The carrier gas was high-purity nitrogen (99.999%). The inlet temperature was 250 °C, the split ratio was 30:1, the detector temperature was 260 °C, the flow rate was 40 mL/min for hydrogen and 300 mL/min for air, and all solvents and samples were filtered through a 0.2 μm filter.

2.6. Statistical Analysis

Multivariate analyses, including PCA, OPLS-DA, and HCA, were conducted using the SIMCA 16.0.2 software package.

Metabolites were annotated and pathway analysed using the KEGG Compound Database (http://www.kegg.jp/kegg/compound/; accessed on 20 January 2024).

3. Results

3.1. Analysis of Physical and Chemical Properties

Table 1 shows the pH, TA, TS, and alcohol concentration of the mixed fermentation MGBW. The pH of the base-wine was 4.11 ± 0.008, with a TA of 5.06 ± 0.069 g/L, TS of 5.80 ± 0.286 g/L, and alcohol concentration of 4.13 ± 0.125 *v*/*v*, %. These values were sufficient for subsequent blending to create a low-alcohol beverage. The total soluble solids (TS) and alcohol concentration were at optimal levels (TS: 5.80 ± 0.286 g/L; alcohol: 4.13 ± 0.125 *v*/*v*, %) for blending into a low-alcohol beverage.

Table 1. Physiochemical indices of Munage Grape Base-Wine.

Physicochemical Indices		Method	Reference
TS (g/L)	5.80 ± 0.286	Direct titration	[26]
TA (g/L tartaric acid)	5.06 ± 0.069	Potentiometric titration	[27]
pH	4.11 ± 0.008	pH meter method	[28]
Alcohol (*v*/*v*, %)	4.13 ± 0.1	Alcohol meter method	[29]

3.2. Volatile Metabolomics Analysis of MGBW

After fermentation, the metabolite profile of each MGBW sample was analysed using HS-SPME-GC-MS. A total of 451 metabolites were identified, including esters, heterocyclic compounds, ketones, hydrocarbons, terpenoids, organic acids, aldehydes, and alcohols. The identified metabolites are listed in Table S1.

The differences in volatile metabolic components and their contents among the samples were analysed using principal component analysis (PCA) (refer to Figure 2a,b). The plot shows that both the mixed fermentation (Mix) and single fermentation (EC1118, TD) samples had highly concentrated individual points, indicating a high level of repeatability. The plot was divided into two regions, and there was a significant separation between the Mix, EC1118, and TD samples. This suggests that the mixed fermentation of the Munage grape base-wine by *Saccharomyces cerevisiae* EC-1118 and *Torulaspora delbrueckii* Bio-119667 had an impact on the volatile metabolite composition in the wine.

The V-plot in Figure 2c,d visualises the metabolites and their compositions in both the mixed fermentation and single fermentation samples. After conducting two pairwise comparisons, it was found that 315 metabolites were statistically significant in distinguishing mixed fermentation wines from EC1118 single fermentation wines. Of these, 132 metabolites increased (red dots) and only 2 decreased (green dots) (Figure 2c,d). Furthermore, a total of 311 metabolites were statistically significant in distinguishing mixed fermentation wines from TD single fermentation wines. As shown in Figure 2d, 135 metabolites increased (red dots) and only 3 decreased (green dots).

Metabolites with similar characteristics were classified using hierarchical cluster analysis to identify intergroup variations (Figure 2e). The order of decreasing metabolites is indicated by the colour sequence from green to red. In comparison to single fermentation wines, mixed fermentation MGBW showed significant upregulation of esters, terpenoids, heterocyclic compounds, aldehydes, ketone metabolites, and their derivatives.

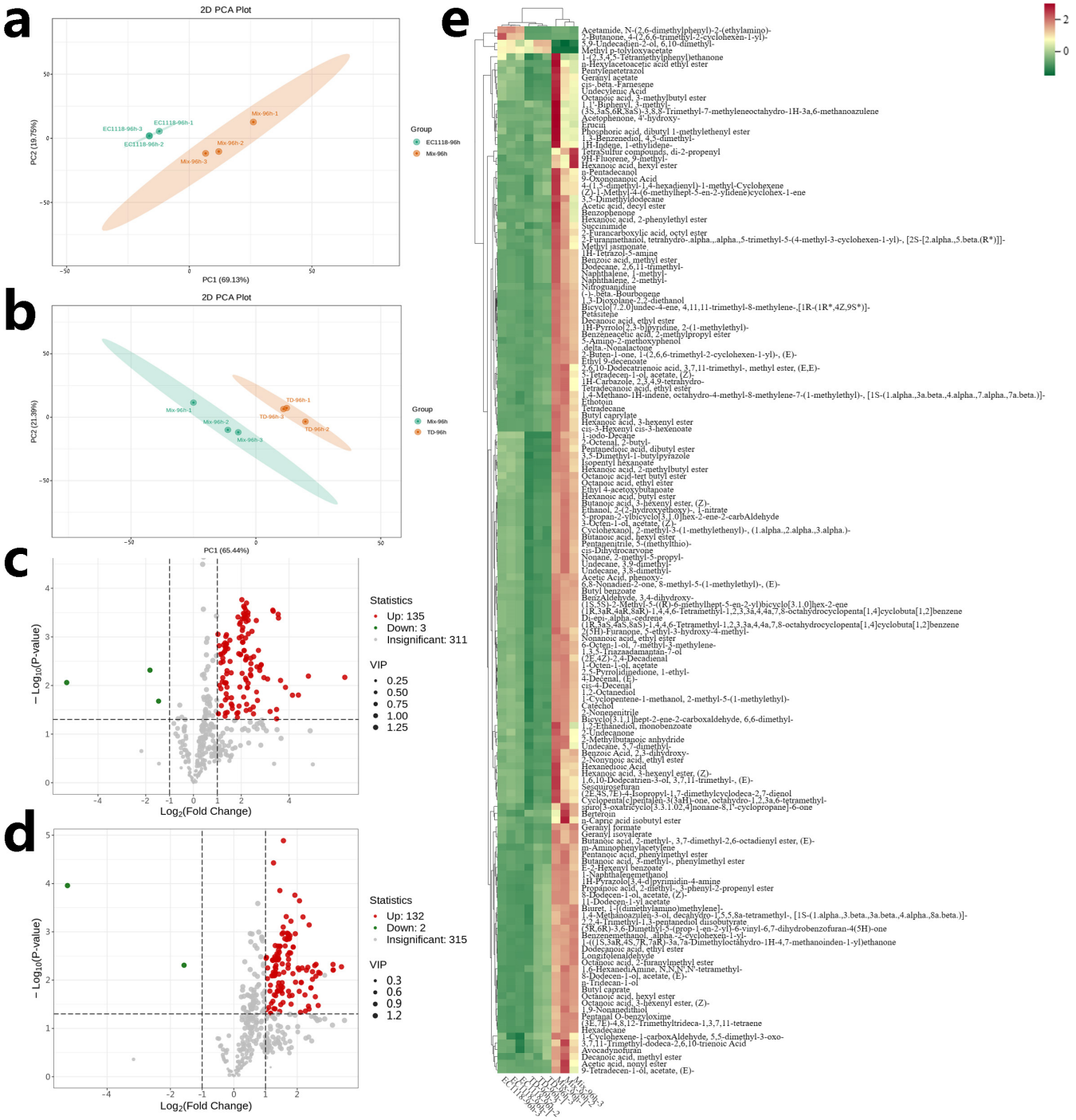

Figure 2. (**a**) Principal component analysis (PCA) of mixed fermentation MGBW (Mix-96h) by single fermentation MGBW (EC1118-96h); (**b**) Principal component analysis (PCA) of mixed fermentation MGBW (Mix-96h) by single fermentation MGBW (TD-96h); (**c**) V-plot of mixed fermentation MGBW (Mix-96h) by single fermentation MGBW (EC1118-96h); (**d**) V-plot of mixed fermentation MGBW (Mix-96h) by single fermentation MGBW (TD-96h); (**e**) The classification heat map of total differential metabolites of three MGBW samples.

3.3. Analysis of MGBW Aroma Composition

After analysing the metabolites in the mixed fermentation MGBW, we aimed to investigate the alterations in volatile metabolite compositions in MGBW after being mixed with EC-1118 and TD. We selected 155 metabolites involved in metabolism for detailed analysis. The metabolites were divided into 15 classes: esters, terpenoids, heterocyclic compounds,

ketones, hydrocarbons, acids, aldehydes, halogenated hydrocarbons, nitrogen compounds, sulfur compounds, phenols, aromatics, alcohols, amines, and others. Table 2 summarises the metabolites with relatively high levels in MGBW with VIP scores > 1. These included esters (55 metabolites), terpenoids (21 metabolites), heterocyclic compounds (13 metabolites), ketones (10 metabolites), hydrocarbons (10 metabolites), acids (6 metabolites), aldehydes (6 metabolites), halogenated hydrocarbons (1 metabolite), nitrogen compounds (4 metabolites), sulfur compounds (2 metabolites), phenols (3 metabolites), aromatics (4 metabolites), alcohols (13 metabolites), amines (3 metabolites), and others (4 metabolites). Metabolites, including 1-iodo-decane; L-aspartic acid, n-acetyl-, dimethyl ester; isopentyl hexanoate; pentanedioic acid, dibutyl ester; 2-nonynoic acid, ethyl ester; benzoic acid, 2,3-dihydroxy-; n-hexylacetoacetic acid ethyl ester; hexanoic acid, 2-methylbutyl ester; butyl benzoate; 6,8-Nonadien-2-one, 8-methyl-5-(1-methylethyl)-, (E)-; acetic acid, decyl ester; hexanoic acid, 3-hexenyl ester, (Z)-; geranyl acetate; geranyl formate, were significantly upregulated after mixed fermentation. However, the production of 5,9-undecadien-2-ol, 6,10-dimethyl-, and methyl *p*-tolyloxyacetate was downregulated in the mixed fermentation MGBW.

Table 2. The VIP value of primary materials in MGBW.

Class	Name	VIP
Amines (3)	N,N,N′,N′-tetramethyl-1,6-HexanediAmine	1.05
	N-(2,6-dimethylphenyl)-2-(ethylamino)-Acetamide	1.15
	3-Aminophenylacetylene	1.23
Alcohols (13)	(2E,4S,7E)-4-Isopropyl-1,7-dimethylcyclodeca-2,7-dienol	1.21
	1,2-Octanediol	1.22
	1,3-Dioxolane-2,2-diethanol	1.19
	2-methyl-5-(1-methylethyl)-1-Cyclopentene-1-methanol	1.18
	1-Naphthalenemethanol	1.09
	1-Octen-1-ol, acetate	1.22
	2-butyl-2-Octenal	1.21
	6,10-dimethyl-5,9-Undecadien-2-ol	1.23
	alpha.-2-cyclohexen-1-yl-Benzenemethanol	1.21
	(1.alpha.,2.alpha.,3.alpha.)-2-methyl-3-(1-methylethenyl)-Cyclohexanol	1.22
	1-nitrate2-(2-hydroxyethoxy)-Ethanol	1.19
	n-Pentadecanol	1.22
	n-Tridecan-1-ol	1.23
Aromatics (4)	3-methyl-1,1′-Biphenyl	1.23
	9-methyl-9H-Fluorene	1.20
	1-methyl-Naphthalene	1.22
	2-methyl-Naphthalene	1.18
Phenols (3)	4,5-dimethyl-1,3-Benzenediol	1.23
	5-Amino-2-methoxyphenol	1.22
	Catechol	1.23
Sulfur compounds (2)	1,9-Nonanedithiol	1.21
	di-2-propenyl-TetraSulfur compounds	1.20
Nitrogen compounds (4)	2-Nonenenitrile	1.23
	1-[(dimethylamino)methylene]-Biuret	1.19
	Nitroguanidine	1.22
	Pentanal O-benzyloxime	1.21
Halogenated hydrocarbons (1)	1-iodo-Decane	1.23
Others (4)	2-Methylbutanoic anhydride	1.03
	Berteroin	1.23
	Erucin	1.22
	5-(methylthio)-Pentanenitrile	1.22
Aldehydes (6)	(2E,4Z)-2,4-Decadienal	1.21
	5,5-dimethyl-3-oxo-1-Cyclohexene-1-carboxAldehyde	1.23

Table 2. *Cont.*

Class	Name	VIP
	(E)-4-Decenal	1.20
	5-propan-2-ylbicyclo[3.1.0]hex-2-ene-2-carbAldehyde	1.22
	3,4-dihydroxy-BenzAldehyde	1.22
	cis-4-Decenal	1.22
Acids (6)	Undecylenic Acid	1.22
	Hexanedioic Acid	1.22
	2,3-dihydroxy-Benzoic Acid	1.21
	phenoxy-Acetic Acid	1.11
	9-Oxononanoic Acid	1.21
	3,7,11-Trimethyl-dodeca-2,6,10-trienoic Acid	1.21
Terpenoids (21)	Petasitene	1.23
	Longifolenaldehyde	1.22
	Geranyl acetate	1.18
	Di-epi-.alpha.-cedrene	1.22
	cis-Dihydrocarvone	1.20
	cis-.beta.-Farnesene	1.19
	[1R-(1R*,4Z,9S*)]-4,11,11-trimethyl-8-methylene-Bicyclo[7.2.0]undec-4-ene	1.23
	6,6-dimethyl-Bicyclo[3.1.1]hept-2-ene-2-carboxaldehyde	1.23
	7-methyl-3-methylene-6-Octen-1-ol	1.21
	4-(1,5-dimethyl-1,4-hexadienyl)-1-methyl-Cyclohexene	1.15
	2-Furanmethanol, tetrahydro-.alpha.,.alpha.,5-trimethyl-5-(4-methyl-3-cyclohexen-1-yl)-,[2S-[2.alpha.,5.beta.(R*)]]-	1.16
	(E)-3,7,11-trimethyl-1,6,10-Dodecatrien-3-ol	1.04
	[1S-(1.alpha.,3.beta.,3a.beta.,4.alpha.,8a.beta.)]-decahydro-1,5,5,8a-tetramethyl-1,4-Methanoazulen-3-ol	1.20
	[1S-(1.alpha.,3a.beta.,4.alpha.,7.alpha.,7a.beta.)]-octahydro-4-methyl-8-methylene-7-(1-methylethyl)-1,4-Methano-1H-indene	1.15
	(Z)-1-Methyl-4-(6-methylhept-5-en-2-ylidene)cyclohex-1-ene	1.21
	(5R,6R)-3,6-Dimethyl-5-(prop-1-en-2-yl)-6-vinyl-6,7-dihydrobenzofuran-4(5H)-one	1.23
	(3S,3aS,6R,8aS)-3,8,8-Trimethyl-7-methyleneoctahydro-1H-3a,6-methanoazulene	1.22
	(3E,7E)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene	1.21
	(1R,3aS,4aS,8aS)-1,4,4,6-Tetramethyl-1,2,3,3a,4,4a,7,8-octahydrocyclopenta[1,4]cyclobuta[1,2]benzene	1.22
	(1R,3aR,4aR,8aR)-1,4,4,6-Tetramethyl-1,2,3,3a,4,4a,7,8-octahydrocyclopenta[1,4]cyclobuta[1,2]benzene	1.23
	(-)-.beta.-Bourbonene	1.21
Hydrocarbons (10)	5,7-dimethyl-Undecane	1.22
	3,9-dimethyl-Undecane	1.21
	3,8-dimethyl-Undecane	1.18
	Tetradecane	1.18
	2-methyl-5-propyl-Nonane	1.10
	Hexadecane	1.23
	2,6,11-trimethyl-Dodecane	1.16
	3,5-Dimethyldodecane	1.22
	1-ethylidene-1H-Indene	1.20
	(1S,5S)-2-Methyl-5-((R)-6-methylhept-5-en-2-yl)bicyclo[3.1.0]hex-2-ene	1.21
Ketones (10)	spiro[3-oxatricyclo[3.3.1.02,4]nonane-8,1′-cyclopropane]-6-one	1.22
	octahydro-1,2,3a,6-tetramethyl-Cyclopenta[c]pentalen-3(3aH)-one	1.15
	Benzophenone	1.22
	4′-hydroxy-Acetophenone	1.22
	(E)-8-methyl-5-(1-methylethyl)-6,8-Nonadien-2-one	1.22
	2-Undecanone	1.22
	(E)-1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-2-Buten-1-one	1.23
	4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-2-Butanone	1.21
	1-(2,3,4,5-Tetramethylphenyl)ethanone	1.17

Table 2. *Cont.*

Class	Name	VIP
	1-((1S,3aR,4S,7R,7aR)-3a,7a-Dimethyloctahydro-1H-4,7-methanoinden-1-yl)ethanone	1.11
Heterocyclic compounds (13)	Succinimide	1.22
	Sesquirosefuran	1.22
	Pentylenetetrazol	1.22
	Ethotoin	1.14
	Avocadynofuran	1.22
	3,5-Dimethyl-1-butylpyrazole	1.22
	1-ethyl-2,5-Pyrrolidinedione	1.10
	5-ethyl-3-hydroxy-4-methyl-2(5H)-Furanone	1.23
	1H-Tetrazol-5-amine	1.22
	2-(1-methylethyl)-1H-Pyrrolo[2,3-b]pyridine	1.23
	1H-Pyrazolo[3,4-d]pyrimidin-4-amine	1.17
	2,3,4,9-tetrahydro-1H-Carbazole	1.20
	1,3,5-Triazaadamantan-7-ol	1.16
Esters (55)	Tetradecanoic acid, ethyl ester	1.22
	2-methyl-Propanoic acid, 3-phenyl-2-propenyl ester	1.19
	Phosphoric acid, dibutyl 1-methylethenyl ester	1.17
	Pentanoic acid, phenylmethyl ester	1.23
	Pentanedioic acid, dibutyl ester	1.13
	Octanoic acid-tert butyl ester	1.23
	Octanoic acid, hexyl ester	1.22
	Octanoic acid, ethyl ester	1.22
	Octanoic acid, 3-methylbutyl ester	1.23
	(Z)-Octanoic acid, 3-hexenyl ester	1.10
	Octanoic acid, 2-furanylmethyl ester	1.20
	Nonanoic acid, ethyl ester	1.21
	n-Hexylacetoacetic acid ethyl ester	1.22
	n-Capric acid isobutyl ester	1.17
	Methyl p-tolyloxyacetate	1.13
	Methyl jasmonate	1.20
	Isopentyl hexanoate	1.10
	Hexanoic acid, hexyl ester	1.23
	Hexanoic acid, butyl ester	1.23
	(Z)-Hexanoic acid, 3-hexenyl ester	1.23
	Hexanoic acid, 3-hexenyl ester	1.22
	Hexanoic acid, 2-phenylethyl ester	1.18
	Hexanoic acid, 2-methylbutyl ester	1.17
	Geranyl isovalerate	1.05
	Geranyl formate	1.22
	Ethyl 9-decenoate	1.21
	Ethyl 4-acetoxybutanoate	1.22
	E-2-Hexenyl benzoate	1.18
	Dodecanoic acid, ethyl ester	1.22
	Decanoic acid, methyl ester	1.23
	Decanoic acid, ethyl ester	1.22
	cis-3-Hexenyl cis-3-hexenoate	1.22
	Butyl caprylate	1.05
	Butyl caprate	1.21
	Butyl benzoate	1.22
	Butanoic acid, hexyl ester	1.11
	3-methyl-Butanoic acid, phenylmethyl ester	1.13
	(Z)-Butanoic acid, 3-hexenyl ester,	1.21
	(E)-3,7-dimethyl-2-methyl-Butanoic acid,2,6-octadienyl ester,	1.21
	Benzoic acid, methyl ester	1.18
	Benzeneacetic acid, 2-methylpropyl ester	1.22
	Acetic acid, nonyl ester	1.23
	Acetic acid, decyl ester	1.22

Table 2. *Cont.*

Class	Name	VIP
	(E)-9-Tetradecen-1-ol, acetate,	1.23
	(Z)-8-Dodecen-1-ol, acetate	1.22
	(E)-8-Dodecen-1-ol, acetate	1.21
	(Z)-5-Tetradecen-1-ol, acetate	1.19
	(Z)-3-Octen-1-ol, acetate	1.23
	2-Nonynoic acid, ethyl ester	1.22
	2-Furancarboxylic acid octyl ester	1.23
	(E,E)-3,7,11-trimethyl-,2,6,10-Dodecatrienoic acid methyl ester	1.20
	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	1.23
	11-Dodecen-1-yl acetate	1.22
	monobenzoate-1,2-Ethanediol	1.18
	.delta.-Nonalactone	1.22

3.4. Dynamics of Key Aroma Components

Figure 3a shows the total relative odor activity value (rOAV) of MGBW that was mixed-fermented by *Saccharomyces cerevisiae* EC-1118 and *Torulaspora delbrueckii* Bio-119667, as well as MGBW that was single-fermented. The rOAV of MGBW fermented with EC-1118 and TD increased significantly by 385% compared to the control. Furthermore, the rOAV of MGBW increased significantly by 205% and 275% when mixed-fermented, respectively, compared to MGBW fermented solely with EC-1118 or TD. To better understand the role of mixed fermentation in enhancing the aroma profile of MGBW, we investigated the changes in key aroma compounds (i.e., relative odor activity value (rOAV) > 1) throughout the fermentation process. A total of 27 key aroma components were selected for in-depth analysis (Table S2). The identified key aroma components included 16 esters, 1 heterocyclic compound, 2 ketones, 1 hydrocarbon, 2 terpenoids, 2 aldehydes, 2 aromatics, and 1 other volatile metabolite.

Figure 3b shows the changes in the relative content of 27 key aroma components over time during fermentation. Compared to the MGBW fermented for 0 h, the MGBW fermented for 48, 72, and 96 h with mixed fermentation showed varying degrees of increase in 27 key aroma compounds. These compounds exhibited a pattern of increasing, decreasing, and then increasing again with fermentation time.

Figure 3c illustrates the changes in the rOAV of the 27 key aroma components during fermentation. The changes in the relative contents of the 27 key aroma components with fermentation time were consistent with the dynamic changes in their rOAV. The trend observed was an initial increase, followed by a decrease, and then another increase.

The trends of rOAV and relative content of these 27 key aroma components with the most significant content changes after mixed fermentation were consistent, and we hypothesise that this trend of these 27 key aroma components, in the case of esters, may be due to the involvement of TD in upregulating the level of fatty acid metabolism in the fermentation system. From this, we can also speculate that the time for one cycle of complete fatty acid metabolism in MGBW during fermentation may be 48 h. This also provides a new idea for us to further explore the effect of TD on the aroma formation of MGBW.

3.5. Pathway Analysis

Figure 4a shows the biosynthetic pathways responsible for the production of key aroma compounds during fermentation. For instance, Figure 4b presents the upregulated volatile metabolites detected in MGBW and the enzymes involved in regulating the metabolites in the methyl jasmonate biosynthetic pathway.

3.6. Pathway Verification

GC was used to detect fatty acids, including α-linolenic acid, in MGBW to verify the speculation that lipid biosynthesis is caused by mixed fermentation involving TD. The content of α-linolenic acid significantly decreased after 96 h of fermentation (Figure 5), indicating fatty acid metabolism during mixed fermentation.

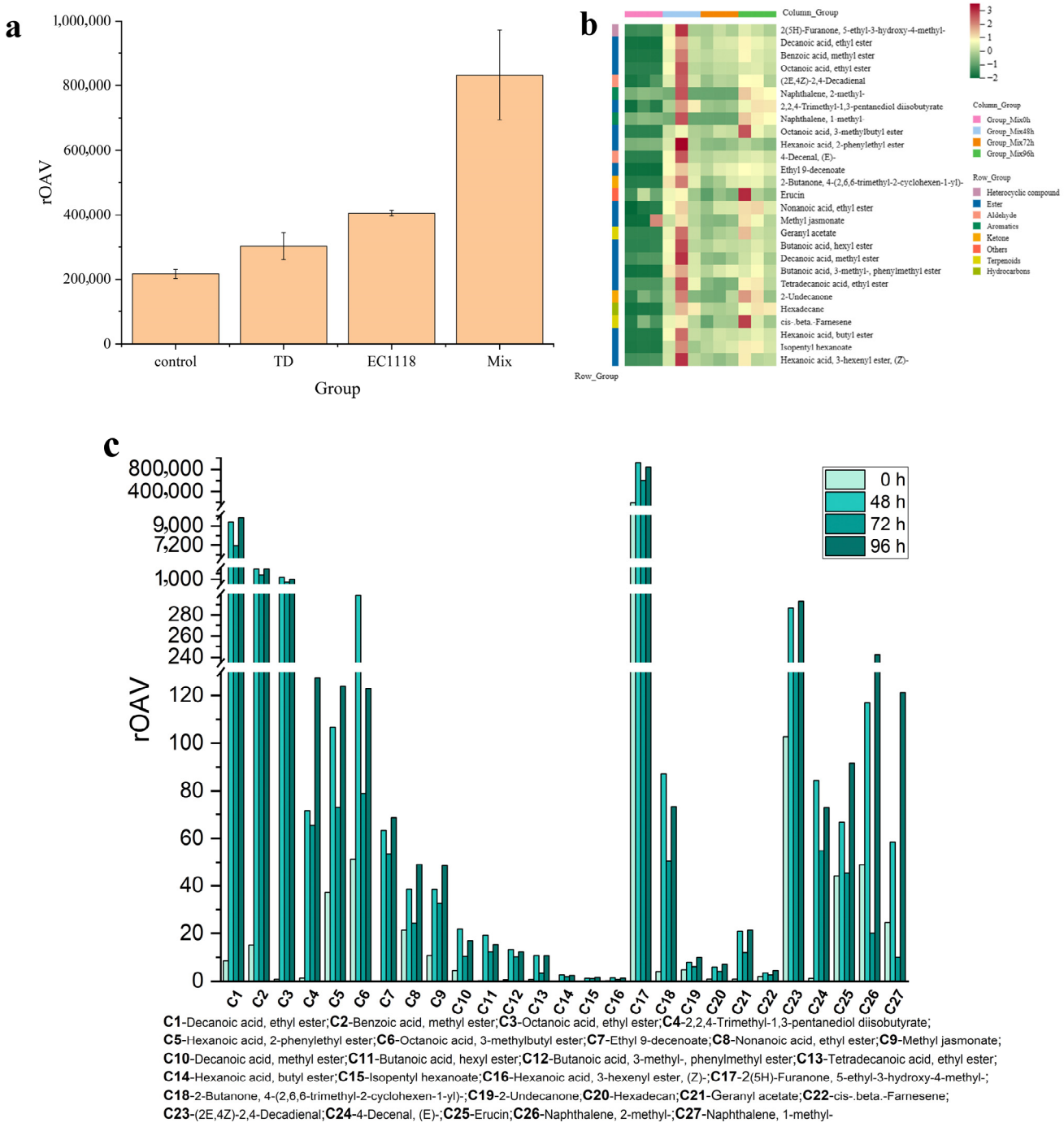

Figure 3. (**a**) The total relative odor activity value (rOAV) of MGBW that was mixed-fermented by EC1118 and TD for 96h, as well as MGBW that was single-fermented; (**b**) the changes in the relative content of 27 key aroma components over time during mixed fermentation; (**c**) the changes in the rOAV of the 27 key aroma components during mixed fermentation.

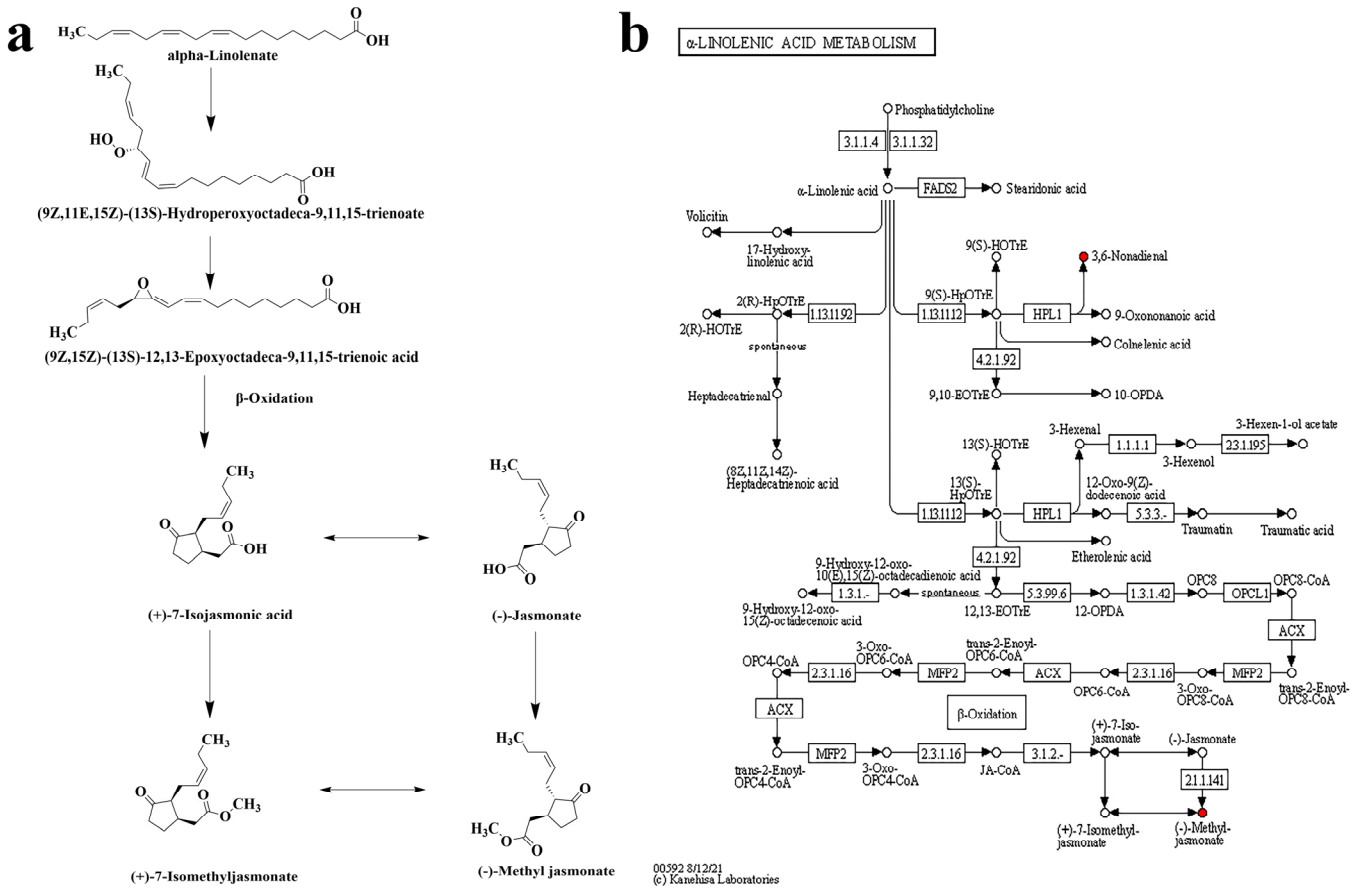

Figure 4. (**a**) Major biosynthetic pathways of esters (using methyl jasmonate as an example); (**b**) the upregulated volatile metabolites detected in MGBW and the enzymes involved in regulating the metabolites in the methyl jasmonate biosynthetic pathway.

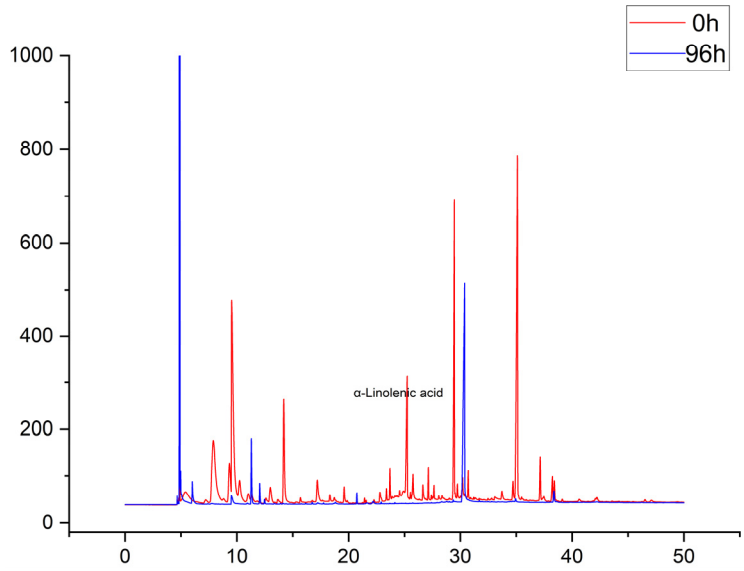

Figure 5. Changes in α-linolenic acid content before and after mixed fermentation.

4. Discussion

4.1. Application of Torulaspora delbrueckii in the Mixed Fermentation of Wine

The complexity of the aroma in autochthonous wines is often attributed to the high proportion of non-Saccharomyces involved in their fermentation [30]. Some scholars

have investigated the use of selected non-Saccharomyces and *Saccharomyces* for mixed fermentation to mimic the microbial community structure of spontaneous fermentation. This can shape the character and typicality of the wine and add complexity to the aroma [31]. Numerous scholars have extensively researched the use of mixed fermentation processes that involve non-Saccharomyces. This enhances the complexity and quality of the aromatic composition of wines. The effectiveness of the mixed fermentation method depends largely on the metabolic pathways, biosynthesis of key aroma components, and the contribution to the wine's aromatic composition by non-Saccharomyces involved in the process [32,33]. The experiment involved the mixed fermentation of *Vitis vinifera* L. cv. *Munage* base-wine using *Torulaspora delbrueckii* Bio-119667. The physicochemical indices measured for MGBW in Table 1 indicate that this non-Saccharomyces yeast is highly suitable and exhibits excellent fermentation performance for Munage grape juice.

In this study, the use of TD for mixed fermentation of Munage grape juice was beneficial for two reasons. Firstly, the strain TD was well suited for fermentation. In our preliminary experiments, we investigated the suitability of 11 different yeast strains for Munage grape juice. After pre-screening, the non-Saccharomyces cerevisiae strains selected for this experiment were well adapted to the high-sugar–acid environment of grape juice. This made it easy for them to grow and produce substances with sufficient aroma. Secondly, we conducted preliminary experiments to optimise the MGBW fermentation process in order to improve the aroma production capacity of TD. This included optimising fermentation time, temperature, inoculum amount, and inoculation sequence.

Overall, the non-Saccharomyces, TD, used in this experiment showed good suitability for Munage grape juice and improved the aromatic composition of MGBW. This provides a basis for the development of aromatic, low-alcohol Munage grape fermented wines.

4.2. Aroma Composition of MGBW

Metabolomics analyses showed that the content of all types of volatile metabolites increased to varying degrees after 96 h of fermentation by a mixture of TD and EC-1118, resulting in a more complex aroma composition of MGBW, as shown in Figure 2e in the results. After analysing the data from the untargeted metabolome, we found that the following classes of compounds present a major contribution in the aroma formation of MGBW.

4.2.1. Esters

Esters are significant components of the aroma of fermented fruit wines. Most of the fruity flavours in fruit wines are produced by esters [34]. Although various esters contribute to the fruit aroma of many fruits, their content is limited. The fruit aroma of fruit wines is mainly formed by secondary metabolism during the yeast fermentation process [35]. These esters can be classified into two main groups based on their structure and synthesis pathways in yeast: acetate esters, which are formed by the degradation products of acetyl coenzyme A and amino acids, and ethyl esters of fatty acids or fatty acid esters, which are formed by the metabolism of fatty acids [36]. Acetate and most of the short- and medium-chain fatty acid ethyl esters have low aroma thresholds and contribute significantly to the aroma of wines, particularly white and rose wines. The metabolomics analysis of this experiment revealed that MGBW contained 115 esters, which accounted for 25.5% of the total volatile metabolites. Additionally, 73 esters with rOAV > 1 were identified, which is an increase of 24 esters compared to Munage grape juice. The most abundant ester was ethyl decanoate, followed by ethyl caprylate, both of which are acetate esters with strong sweet and fruity aromas. These esters reached their maximum value after 48 h of fermentation. After 48 h of fermentation, the maximum content of important ethyl esters in fruit wines, including ethyl laurate, ethyl β-phenylacetate, ethyl 9-decenoate, isoamyl caprylate, isoamyl acetate, 2-methylbutyl acetate, and hexyl butyrate, was reached. The present study is in agreement with previous research that TD has a higher capacity to

produce ethyl octanoate. It can be inferred from the text that TD plays a significant role in the production of ester flavours during mixed fermentation.

4.2.2. Alcohol

The production of higher alcohols is a crucial aspect of yeast alcoholic fermentation [37]. The composition of the must's amino acids significantly affects the higher alcohol composition post-fermentation. Advanced alcohols are also known as hetero-alcoholic oils. As most of these substances have a strong, irritating and undesirable odour, they can adversely affect the overall flavour profile of the fruit wine, reducing its quality and leading to a rough, lacklustre aroma. The study found that MGBW contained 39 higher alcohols, which accounted for 8.6% of the total volatile metabolites. However, only six higher alcohols had rOAV > 1, with the highest being 1-nonanol at 86.21. 1-Nonanol, known for its pleasant rose and orange aroma, significantly contributed to the strong floral and fruity aroma of MGBW.

4.2.3. Terpenes

Terpenes and norisoprenoids in wines are derived from grape raw materials and contribute to varietal aroma [38]. These substances present floral and fruity aromas and have a low aroma threshold, directly enhancing the wine's aroma. Their synergistic effects further enhance the wine's aroma. The metabolomic analysis of this experiment revealed that MGBW contained 70 terpenoids, which accounted for 15.5% of the total volatile metabolites. Terpenoids were detected as the second most important class of aroma substances in MGBW. Additionally, 11 terpenes with rOAV > 1 were found. The most abundant terpene was β-bourbonene, a terpene compound with a strong floral aroma. After 48 h of fermentation, the fruit wines had high levels of important ethyl esters, including linalool, α-violetone, levobornone, geranyl acetate, and 1,3,3-trimethylbicyclo[2.2.1]heptan-2-ol. Although most of the other terpenes did not exceed their aroma thresholds, the coexistence of a rich variety of terpenes would collectively contribute positively to the aroma of MGBW through synergistic effects, increasing the complexity of the MGBW aroma.

4.2.4. Heterocyclic Compound

The study detected 61 heterocyclic compounds in MGBW, which accounted for 13.5% of the total volatile metabolites. This is an important class of aroma substance in MGBW. Additionally, five heterocyclic compounds with rOAV > 1 were detected. The heterocyclic compound with the highest rOAV was 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone, which has sweet, fruity, caramel, maple, nutty, and butterscotch aromas. Its rOAV was as high as 773,215.11. The relative content of 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone reached its maximum value after 48 h of fermentation and maintained the same order of magnitude even after 96 h of fermentation. This compound is an important aroma component of MGBW and contributes greatly to its overall aroma composition.

4.2.5. Aldehyde

The study detected 26 aldehydes in MGBW, which accounted for 5.7% of the total volatile metabolites. Among them, 10 aldehydes had rOAV > 1. The aldehyde with the highest rOAV was 2-nonenal, which has a fatty, green, waxy, cucumber, and melon aroma, with an rOAV of 5827.90. The next highest aldehydes were (Z)-6-nonenal and 3,6-nonadienal, which provided green, cucumber, melon, cantaloupe, and honeydew flavours to MGBW.

4.3. Improvement of MGBW Aroma Composition by TD

After PCA, the total variance explained by the two principal components of the mixed and EC-1118 single fermentations was 88.88%, including 69.13% for PC1 and 19.75% for PC2 (Figure 2a,b), which indicated that the mixed fermentation had a significant effect on the composition of the MGBW aroma substances. Based on a broadly targeted metabolomics analysis, 55 esters were identified as differentially volatile metabolites (VIP > 1), accounting

for 32.2% of the total. This suggests that TD may improve the aroma composition of MGBW by modulating ester biosynthesis during fermentation.

The aim of this study was to investigate the metabolism (rOAV) of volatile metabolites such as esters, which play an important role in contributing to the aroma composition of MGBW, in order to improve the aroma profile of MGBW during the fermentation of MGBW involving TD (Figure 2e). Based on the results of the metabolomic analyses of the broad target of MGBW, combined with information from the KEGG database, we conclude that the biosynthetic pathway of esters in MGBW mainly involves the metabolism of fatty acids in the Munage grape juice. In addition, based on the metabolomics analyses and information from the KEGG database, we identified the pathway and synthetic precursors of methyl jasmonate as an example.

Metabolomics analysis detected that the main metabolic pathway for metabolite production during fermentation was the β-oxidation pathway of fatty acids [39,40]. Following mixed fermentation, the content of each key aroma component increased to varying degrees. The biotransformation reactions in this pathway involve several enzymes, as shown in Figure 4b. These include secretory phospholipase A2 [EC:3.1.1.4], HRAS-like suppressor 3 [EC:3.1.1.32 3.1.1.4], lipoxygenase [EC:1.13.11.12], and hydroperoxide dehydratase [EC:4.2.1.92]. The transformation of their metabolic precursors resulted in a significant increase in the content of key aroma compounds of esters, represented by methyl jasmonate, compared to MGBW fermented by a single yeast. The increase in production during fermentation helps to reflect the improvement of the aroma composition of MGBW by the mixed fermentation involving *Torulaspora delbrueckii* Bio-119667. This is because these substances have various aroma profiles.

To test the hypothesis that these esters are synthesised via the α-linolenic acid metabolic pathway during fermentation, we conducted a validation study to determine the biosynthetic pathway of the esters during MGBW fermentation. Figure 5 shows a significant decrease in the content of α-linolenic acid after 96 h of mixed fermentation. These results support our hypothesis that the biosynthesis of key aroma components, specifically esters, in the aroma composition of mixed-fermented MGBW is linked to the metabolism of fatty acids in the musts of Munage grapes. Therefore, these findings provide a stronger theoretical basis for studying the mechanism of mixed fermentation to enhance the aroma composition of MGBW.

5. Conclusions

This study used extensively targeted metabolomics analyses to characterise the effect of mixed fermentation with the addition of TD on the aroma composition of Munage grape base-wine (MGBW). The mixed-fermented MGBW contained a significantly higher amount of esters, terpenoids, and other substances that are important for the aroma composition of wine compared to the monomicrobial-fermented Munage grape juice. This reflects the improvement of the aroma composition of MGBW due to the participation of *Torulaspora delbrueckii* Bio-119667 in the mixed fermentation process. This study is the first to analyse the dynamic changes in and biotransformation mechanisms of aroma composition and key aroma components in mixed fermentation MGBW. The main reaction involved in the metabolite biotransformation of ester aroma components is the β-oxidation of fatty acids, but the exact mechanism of metabolic transformation remains unclear. Future studies will investigate the relationship between the inherent enzymes and metabolites of TD more rigorously. This study provides a theoretical basis for developing wine products with greater aromatic intensity and complexity from the Munage grape.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10050266/s1, Table S1. All simple data; Table S2. Key aroma components and their rOAVs.

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