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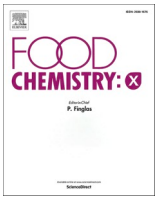

Evaluating of effects for the sequence fermentation with *M. pulcherrima* and *I. terricola* on mulberry wine fermentation: Physicochemical, flavonoids, and volatiles profiles

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ABSTRACT

This study investigates the variation of physicochemical, flavonoids, and volatiles during sequential fermentation which *Metschnikowia pulcherrima* and *Issatchenkia terricola* as sequential co-fermenters and a single fermentation by *Saccharomyces cerevisiae* in mulberry wine. Sequential fermentation shown that β-glucosidase activity greater and fermentation time declined to 144 h. In addition, 11 flavonoids (apigenin-5-O-glucoside, aromadendrin-7-O-glucoside, kaempferol-3,7-O-diglucoside, and so on) were significantly increased. Significant differences were found between types of metabolic products enriched in flavone and flavonol biosynthesis and anthocyanins biosynthesis, with an enrichment ratio of 46.15 % and 23.08 %, respectively. 16 apple-scented compounds (2-Buten-1-one, (E)-1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, Butanoic acid, (Z)-3-hexenyl ester, 3-methyl-1-methylethyl-Butanoic acid ester, and so on), 5 rose-scented (e.g. benzyl alcohol, ethyl geranate, hydrocinnamic acid), and 4 balsamic-scented compounds ((−)-myrtenol, benzoic acid 1-methylethyl ester, benzyl alcohol, p-cymen-7-ol) were distinctively present. Interestingly, tryptophan metabolism and indole alkaloid biosynthesis are only enriched in sequential fermentation.

1. Introduction

Mulberries (*Morus nigra.* L), among the distinctive fruits flourishing in Xinjiang, inherently possess a certain level of fruity aroma and are rich in a plethora of bioactive compounds (Jiang & Nie, 2015). Notably, sweet and aromatic constituents, along with flavonoid compounds, stand out as prominent attributes within mulberries (Skrovankova et al., 2022). Nevertheless, mulberries are perishable because of their short shelf life. They are often processed into mulberry wine through microbial fermentation. Current research have shown that antioxidant activity, anthocyanin, chemical, and so on of mulberry wine is influenced by the type of yeast used for inoculation (Guo et al., 2024) (S. Liu et al., 2017). Therefore, it is necessary to research different yeast inoculation strategies to promote the growth of the mulberry processing industry.

Amidst the diverse array of yeast strains, *Saccharomyces cerevisiae*, commonly employed in the fermentation of fruit wines, not only contributes significantly to the alcohol content of mulberry wine but also imparts a rich tapestry of aromas and essential nutrients (Fairbairn et al., 2017); (Y. Qin et al., 2023). Our previous research has indicated that the aroma profile and nutrient richness of mulberry wine produced solely with conventional *Saccharomyces cerevisiae* have the potential to be further improved (Y. Qin et al., 2024). Consequently, inoculating non-*Saccharomyces cerevisiae* strains during wine fermentation has emerged as a pivotal strategy. Contemporary studies have revealed that a multitude of novel, as yet untapped non-*Saccharomyces cerevisiae* strains may achieve this by generating a diverse array of aromatic active compounds (e.g., terpenes, volatile esters, and phenolic compounds, etc.), while simultaneously exerting beneficial effects on reducing alcohol content, modulating acidity, and enhancing aroma complexity in wines (C. Liu et al., 2022); (Ge et al., 2022) (Canonico et al., 2019). For instance, *Metschnikowia pulcherrima* is renowned for its capacity to produce distinctive aromatic compounds (Binati et al., 2023). Notably, it enhances the production of fruity and floral aromas while concurrently generating polysaccharides and glycerol, thereby augmenting the final product's mouthfeel, rendering it fuller and smoother (Binati et al., 2023) (Sadoudi et al., 2017). Furthermore, it demonstrates commendable efficacy in reducing alcohol content, and acidity, and mitigating the risk of spoilage microorganisms (e.g., acetic acid bacteria) (Windholtz

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et al., 2023). Co-inoculation with *Metschnikowia pulcherrima* also mitigates the risk of viscosity or slow fermentation during the fermentation process, thereby contributing to enhanced overall fermentation stability (Varela et al., 2017). During the fermentation process, polyphenols within the fruit juice are typically glycosylated, and under the influence of various environmental factors (e.g., pH and total sugar), the production of β-glucosidase by *Issatchenkia terricola* can result in the release of phenolic compounds from their glycosidic conjugates, which in turn promotes the production of polyphenol content (e.g., flavonoid) in wine (Jiang et al., 2022). However, *Saccharomyces cerevisiae* and the above two non-*Saccharomyces cerevisiae*'s impact on the complexity of aromas and the fluctuation in flavonoid compound content, improve quality mulberry wine, remain yet to be elucidated.

Thus, volatile metabolomics and comprehensive targeted metabolomics were employed to undertake a comparative analysis between single fermentation with *Saccharomyces cerevisiae* and sequential fermentation with *Metschnikowia pulcherrima* and *Issatchenkia terricola*, focusing on the distinct characteristics about physicochemical, volatiles, and flavonoids. Furthermore, a comprehensive investigation was undertaken to ascertain the underlying factors responsible for these observed differences. This endeavour served to elucidate the essence of the interplay among yeast species during the alcoholic fermentation process of mulberries.

2. Material and methods

2.1. Chemicals and strains

Water was purchased from Wahaha (Zhejiang, China). Carbinol (>98 %, Merck, USA), Acetonitrile (>98 %, Merck, USA), Formic acid (>98 %, Aladdin, Shanghai, China), NaCl (Guoyao, Beijing, China), n-hexane (Merck, USA). Phenyl-polydimethylsiloxane and other standard products used for GC–MS were purchased from BioBioPha/Sigma-Aldrich (USA). *Saccharomyces cerevisiae* DV 10 was purchased from (Lallemand Health Solutions Inc., Montreal, Canada) *Metschnikowia pulcherrima* (bio-119,683) and *Issatchenkia terricola* (BNCC141882) were respectively supplied by Baio Bowei Biotechnology Co., LTD (Beijing, China) and BCNN (Henan, China).

2.2. Preparation of mulberry slurry

The mulberry was harvested in May 2023 from the plantation which is located in the Gaochang district of Turpan City, Xinjiang Province. Then mulberry samples were cryogenically transported and mashed immediately. Subsequently, the mulberry slurry was aseptically dispensed into sterile bottles for further fermentation. Na_2SO_3 (0.12 g/L) was added to the mulberry slurry to eliminate the influence of wild microflora from the raw fruit. Meanwhile, 22.3 mg/L pectinase (LALLEMAND, French) and 0.3 g/L yeast activator (GO-FERM) were also added to the slurry.

2.3. Fermentation process of mulberry slurry

YPD medium (10 g/L yeast extract, 20 g/L glucose, 20 g/L peptone, and 20 g/L agar) was utilized for incubation of *S. cerevisiae*, *M. pulcherrima,* and *I. terricola*. Subsequently, two distinct fermentation processes were initiated: Single fermentations (S) by *Saccharomyces cerevisiae* DV 10 and sequential mixture fermentation (M) by *S. cerevisiae* DV 10, *M. pulcherrima* (bio-119,683) and *I. terricola* (BNCC 141882). The strains were inoculated into mulberry supernatant slurry with the initial concentration of 1×10^6 CFU/mL. For SF 0.15 g/L yeast nutrient (FERMAID E) was added at 24,48 and 96 h. For M, *S. cerevisiae* DV was inoculated first, then *M. pulcherrima* inoculated at 48 h followed by *I. terricola* inoculation at 96 h, contemporaneous added 0.15 g/L yeast nutrient (FERMAID E). A total of 5.0 L mulberry supernatant slurry was split into a 10 L wide-mouth reagent bottle for each treatment. During the fermentation process, the total sugar was kept to 23.0 % (*w*/w) by adding sucrose. Both fermentation were carried out at 22 ± 1 °C until the content of alcohol was 7 *v*/*v* % and the physicochemical properties, flavonoid compounds, and volatile constituents were analyzed to characterize S and M specificity.

2.4. Monitoring of physicochemical properties

The measurement methods of total sugar (TS), total soluble solids (Brax), ethanol content (% *v*/*v*), and pH value were consistent with our previous studies. Yeast assimilates nitrogen was determined by rapid measurement (Formaldehyde process). β-glucosidase activity was monitored by a test kit (Solarbio Science & Technology Co., Ltd., Beijing, China).

2.5. Volatile profile analysis

Materials were gathered, weighed, immediately frozen in liquid nitrogen, and kept at −80 °C for later use. Subsequently, the samples were pulverized in liquid nitrogen. A 500 mg (1 mL) aliquot of the powdered material was rapidly transferred to a 20 mL headspace vial (Agilent, Palo Alto, CA, USA) containing a NaCl-saturated solution to arrest enzymatic activity. The vials were securely sealed using crimp-top caps fitted with TFE-silicone septa (Agilent). Prior to SPME analysis, each vial was preheated at 60 °C for 5 min, followed by a 15-min exposure of a 120 μm DVB/CWR/PDMS fiber (Agilent) to the sample headspace at 60 °C.

After processing the sample, our previous studies have described the specific operating parameters of HS-SPME-GC–MS (Y. Qin et al., 2024).

2.6. Flavonoid profile targeted analysis

Firstly, 100 μL of the sample was combined with 100 μL of 70 % methanol (1,1 *V*/V ratio) and vortexed for 15 min. Subsequently, centrifugation was performed at 12,000 rpm for 3 min at 4 °C (Eppendorf 5424R centrifuge). The supernatant was filtered (by 0.22 μm filter) and analyzed using a UPLC-ESI-MS/MS system (UPLC, ExionLC™ AD) coupled with a tandem mass spectrometry system. The analytical conditions were as follows: the UPLC column was an Agilent SB-C18 (1.8 μm, 2.1 mm × 100 mm). The mobile phase comprised solvent A (pure water with 0.1 % formic acid) and solvent B (acetonitrile with 0.1 % formic acid). The gradient program initiated (95 % A and 5 % B), linearly transitioning to 5 % A and 95 % B within 9 min, and maintaining this composition for 1 min. Thereafter, the composition was adjusted (95 % A and 5 % B) within 1.1 min and maintained for 2.9 min. The flow rate: 0.35 mL/min, the column oven temperature: 40 °C, and the injection volume: 2 μL. Next, the effluent was directed alternately to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS for analysis.

The ESI source's operating parameters are optimized as follows: the source temperature: 500 °C, with the ion spray voltage (IS) set to positive ion mode: 5500 V) and negative ion mode: −4500 V. The ion source gas I (GSI): 50 psi, gas II (GSII): 60 psi, and curtain gas (CUR): 25 psi, to ensure efficient ionization and transmission of ions. The collision-activated dissociation (CAD) was set to high to enhance the fragmentation of analyte ions. For quantitative analyses, QQQ scans were acquired in multiple reaction monitoring (MRM) mode with the collision gas (nitrogen) set to medium. The dephasing potential (DP) and collision energy (CE) of each MRM transition were optimized to maximize the sensitivity and specificity of the assay. A unique set of MRM transitions is monitored for each metabolite eluted in each chromatographic cycle, ensuring accurate and reliable quantification of the target analyte.

2.7. Metabolites annotation and enrichment analysis

Identified metabolites were annotated by cross-referencing them against the KEGG compound database (www.kegg.jp/kegg/compound/). Thereafter, they were mapped to the KEGG Pathway database (www.

kegg.jp/kegg/pathway.html). which provides a comprehensive overview of metabolic networks and pathways in various organisms. Pathways that exhibited significant regulation of mapped metabolites were then subjected to metabolite sets enrichment analysis (MSEA). This analysis evaluated the enrichment of metabolite sets within the significantly regulated pathways, providing a statistical measure of their importance and significance. The significance of the enrichment was determined using hypergeometric test's *p*-values, which allowed for the identification of those pathways that were most strongly associated with the observed metabolite changes.

2.8. Date and statistical analysis

MassHunter software was used for mass spectrometric analysis, raw data was then extracted and volatile compounds were annotated by a custom-built library (MetWare, Hubei, China). The accurate volatile compounds data was achieved through TIC overlap, CV distribution graphs, and RI. For the targeted determination of flavonoid compounds, the MetWare Database (Metware Biotechnology Co., Ltd., Hubei, China) was utilized, leveraging secondary spectrum information for substance qualification. During analysis, isotope signals, redundant signals containing K^+, Na^+, $NH4^+$, as well as duplicated signals originating from fragments of larger molecular substances, were eliminated. Subsequent software Analyst 1.6.3 (ABSCIEX, American) was employed for the processing of mass spectrometric data. Basic data analysis charts were utilized through Origin 2021pro. SPSS software and Excel 2020 was used for significance analysis. The different correlation analysis was plotted using websites:https://www. omicstudio. cn/tool and https://cloud.metware.cn/#/tools.

3. Result and discussion

3.1. Physicochemical parameter analysis

The physicochemical parameters of different fermentation strategies were presented in Table 1. The results indicate Total soluble solids significant decrease ($P < 0.05$) as fermentation progresses under both strategies. At the end of fermentation, the soluble solids content in the S stands at 9.26 %. Brix, representing a reduction of 3.9 % compared to the initial juice, and similar to the findings in the M. The overall variation in total sugar content was consistent with the trend observed in soluble solids. It was noted that the S exhibited the lowest total sugar content (0.19 g/L), which was 69 % lower compared to the M at the end of fermentation. This suggests that within the 'M' group, the introduction of *M. pulcherrima* and *I. terricola* at 48 and 96 h, respectively, may have imparted a heightened capacity for sugar consumption. Regarding pH, similar patterns were noted in both 'S' and 'M' (Endpoint: 4.08 and 4.06, respectively, $P < 0.05$). This phenomenon is due to the use of carbohydrates by microorganisms in the fermentation process to produce organic acids (e.g., lactic acid and acetic acid) (Punia Bangar et al., 2022). Yeast assimilable nitrogen (YAN) plays a pivotal role in regulating yeast biomass formation and fermentation rate (Li et al., 2024). Throughout the entire fermentation process, there was a decline (S: 0.055 mg/L; M: 0.172 mg/L) in the yeast assimilable nitrogen content, signifying its utilization for yeast growth (Ilaria & Marco, 2021). The activity of β-glucosidase in the 'S' (65.58 μmol/ min) had decreased by 53 % compared to the commencement of fermentation at the conclusion of fermentation. Conversely, in the 'M', the enzyme activity (299.97 μmol/ min) at the end of fermentation had risen by 83.7 % in contrast to its initial levels. This indicates that non-*Saccharomyces cerevisiae* could greatly improve the activity of β-glucosidase during fermentation. Setting the fermentation endpoint at an alcohol content of 7 (% *v/v*), it becomes evident that the inclusion of non-*Saccharomyces cerevisiae* expedited the attainment of the fermentation endpoint for the 'M', reducing the time required to reach this endpoint (S: 144 h; M: 96 h).

3.2. Flavonoid metabolites

Our previous studies have shown that flavonoids were considered as biomarkers to evaluate quality indicators in brewing mulberry wine with *S. cerevisiae* as starter culture (Y. Qin et al., 2023).

3.2.1. Multivariate statistical analysis

Data sets of metabonomic multivariate statistical analysis, including PCA and OPLS - DA model. PCA and QC samples at different stages of the mulberry fermentation process were shown in Supplementary Fig. S1A. All samples and QCs were within 95.00 % confidence intervals. This result explains 53.58 % of the variance between the samples. The fermentation samples of FSS (Flavonoid-Single fermentation-Early) vs. FSL (Flavonoid-Single fermentation-Later), FSS vs. FML (Flavonoid-Sequential fermentation-Later), and FSL vs. FML were separated, which showed significant differences in metabolites between samples. In the OPLS-DA model, three groups were distinguished (Supplementary Fig. 1B—D). The score chart shows excellent model parameters (FSL vs. FSS: $R^2Y = 1$, $Q^2 = 0.954$; FSS vs. FML: $R^2Y = 1$, $Q2 = 0.944$, FML vs. FSL: $R^2Y = 0.999$, $Q^2 = 0.861$) (Supplementary Fig. S2A—C). So OPLS-DA model could reflect the difference in flavonoid compounds during mulberry wine fermentation. VIP, *p*-value, and fold-change were used to identify differential flavonoids in flavonoids in different modes. According to VIP > 1, $P < 0.05$, in positive and negative ion patterns, 137 flavonoids were identified (Table S1). Meanwhile, hierarchical cluster analysis (HCA) was used to compare the contents of flavonoids (Supplementary Fig. S3).

Table 1
Changes in physicochemical indexes under different fermentation strategies.

Physicochemical parameter	0 h		24 h		48 h		96 h		144 h		192 h	
	Single	Mix	Single	Mix	Single	Mix	Single	Mix	Single	Mix	Single	Mix
pH	4.87 ± 0.012 $_a$	4.85 ± 0.012$_a$	4.41 ± 0.023 $_b$	4.48 ± 0.029 $_b$	3.97 ± 0.012 $_f$	3.97 ± 0.012 $_e$	4.36 ± 0.006 $_c$	4.28 ± 0.006 $_c$	4.16 ± 0.006 $_d$	4.06 ± 0.011 $_d$	4.08 ± 0.005 $_e$	–
Brax (%)	15.3 ± 0.289 $_a$	14.88 ± 0.104$_a$	15.1 ± 0.144 $_a$	14.83 ± 0.144 $_a$	10.08 ± 0.145 $_c$	9.85 ± 0.132 $_b$	8.46 ± 0.058 $_e$	8.98 ± 0.637 $_c$	10.81 ± 0.029 $_b$	9.85 ± 0.050 $_b$	9.26 ± 0.017 $_d$	–
TS (g/L)	1.72 ± 0.176 $_c$	1.98 ± 0.087$_b$	3.39 ± 0.589 $_a$	2.85 ± 0.358 $_a$	2.65 ± 0.352 $_b$	2.82 ± 0.196 $_a$	0.53 ± 0.025 $_{de}$	0.22 ± 0.015 $_d$	0.92 ± 0.040 $_d$	0.62 ± 0.017 $_c$	0.19 ± 0.010 $_e$	–
YAN (mg/L)	0.321 ± 0.026 $_b$	0.336 ± 0.039$_a$	0.321 ± 0.013 $_b$	0.324 ± 0.009 $_a$	0.31 ± 0.023 $_b$	0.295 ± 0.006 $_a$	0.232 ± 0.013 $_c$	0.247 ± 0.023$_b$	0.247 ± 0.025 $_c$	0.172 ± 0.013 $_c$	0.055 ± 0.010 $_a$	–
Alcohol (*v/v*, %)	n.d.	n.d.	0.12 ± 0.03 $_{de}$	0.07 ± 0.060 $_d$	0.28 ± 0.025 $_d$	0.25 ± 0.050 $_c$	4.67 ± 0.150 $_c$	5.13 ± 0.120 $_b$	5.03 ± 0.060 $_b$	7.07 ± 0.110 $_a$	7.33 ± 0.290 $_a$	–
β-G (μmol/ min)	139.83 ± 0.37 $_d$	163.29 ± 2.208 $_e$	277.97 ± 0.01$_a$	278.17 ± 1.006 $_b$	194.53 ± 0.098 $_c$	194.48 ± 0.196 $_c$	267.12 ± 0.112 $_b$	166.72 ± 0.295 $_d$	16.1 ± 0.104 $_e$	299.97 ± 0.555 $_a$	65.58 ± 0.271 $_f$	–

Note: Data are presented as mean ± standard deviation ($n = 3$); Single: Single fermentation strategy; Mix: sequence fermentation strategy; a-f: Different lowercase letters indicated significant differences between the samples ($P < 0.05$, one-way ANOVA); TS: total sugar; YAN: Yeast assimilates nitrogen; β-G: β-glucosidase activity; n.d.: not detected.

3.2.2. Differences in flavonoids

Of all the flavonoids (137) generated in different fermentation strategies, the relative content top 15 was identified as different biomarkers (Fig. 1). For example, cyanidin-3-O-glucoside, luteolin-7-O-glucoside, cyanidin-3-O-galactoside, kaempferol-4'-O-glucoside, and so on. Among them, the relative content of 11 flavonoid compounds was significantly varied in comparing M and S. Kaempferol-4'-O-glucoside and kaempferol-3, 7-O-diglucoside were glycosides that bind to flavonols and were susceptible to extracellular enzymatic hydrolysis. As one of the extracellular enzymes, β-glucosidase activity in FML was higher than that in FSL under the action of non-*Saccharomyces cerevisiae* (T. Qin et al., 2021). Therefore, the relative content of kaempferol-4'-O-glucoside and kaempferol-3, 7-O-diglucoside in FML was much higher than that in FSL. This suggests that the addition of *M. pulcherrima* and *I. terricola,* may played a positive role in promoting flavonoid relative content in sequence fermentation strategy. Notably, this is the first time that the sequential fermentation of mulberry wines with *M. pulcherrima* and *I. terricola* has revealed their elevated.

3.2.3. Correlation between flavonoids and physicochemical properties

pH, alcohol content, total sugar, and β-glucosidase (β-G) play crucial roles in the production, transformation, and enrichment of flavonoids (Wei et al., 2022) (Y. Qin et al., 2023). The correlation between them and major flavonoids (abundance top 10) was explored in this study (Fig. 2). A strong positive correlation was shown via the impact of β-glucosidase activity and alcohol content on different major flavonoids selected under different fermentation strategies ($P < 0.05$, Fig. 2A). Additionally, pH had a negative impact on most major flavonoids selected under both strategies (Fig. 2B). Those may indicate, as the alcoholic fermentation process advances, it was evident that flavonoid compounds gradually were enriched. Through hydrogen bonding, hydrophobic interactions, and electrostatic interactions, stable flavonoid-β-glucosidase complexes form, demonstrating the affinity between these entities (Ding et al., 2022). However, as the pH progressively decreases, acidic stress was exerted on the complexes, inducing their transformation back into free monomers. Due to their inherent susceptibility to oxidation, there was a likelihood of a relative reduction in both the quantity and variety of these compounds (Speisky et al., 2022). Additionally, the impact of total sugars on the primary flavonoid compounds differs between the two fermentation strategies. In sequence fermentation, a negative correlation was observed between total sugars and flavonoid compounds, whereas in single fermentation, a positive correlation exists ($P < 0.05$, Fig. 2A). This phenomenon can be attributed to the robust utilization of sugars by yeast strains, substrate competition, and their efficient growth and metabolic capabilities (Koh et al., 2024). Overall, the impact of various fermentation strategies on the correlation between hair flavonoids and physicochemical parameters was comparable.

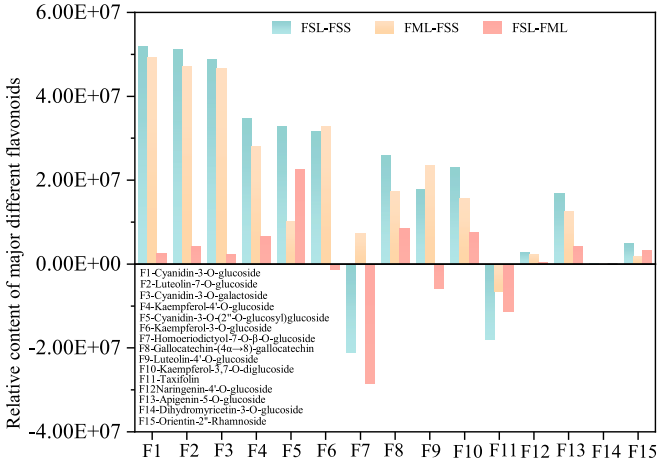

Fig. 1. Changes in flavonoid metabolite relative content in S and M (Differences in the relative content of flavonoid metabolites (Top 15) in different groups (FSL vs. FSS; FML vs. FSS; FML vs. FSL)).

3.2.4. Flavonoids metabolism pathways analysis

In the realm of flavonoid compounds, both the S and M groups exhibit an overlap in flavone and flavonol biosynthesis, anthocyanin biosynthesis, flavonoid biosynthesis, secondary metabolite biosynthesis, and metabolic pathways enriched by KEGG (Supplementary Fig. S4A and B). In terms of quantitative characterization, the S group demonstrates a higher enrichment proportion in the flavonoid biosynthesis at 53.85 % compared to the M. Conversely, the M group surpasses the S group in the proportions of flavone and flavonol biosynthesis, as well as anthocyanin biosynthesis (M: 46.15 %, 23.08 %; S: 30.77 %, 15.38 %).

In flavonoids biosynthesis and flavone and flavonol biosynthesis, apiin (significantly up-regulated by 2.20-fold), lonicerin (significantly up-regulated by 2.03-fold), and dihydrokaempferol (significantly up-regulated by 1.85-fold) were increasingly accumulated with significantly decrease of cosmosiin (2.79-fold), luteolin 7-O-glucoside (2.33-fold), and prunin (2.40-fold) (Supplementary Table S1, Fig. 3). Additionally, homoeriodictyol and luteoforol, as end product in flavonoids biosynthesis under the catalysis of flavanone-3-hydroxylase, flavonoid 3′,5′-hydroxylase (Zhu et al., 2024) (Zhao et al., 2020), and so on, significantly up-regrated 3.78-fold and 1.79-fold (Supplementary Table S1), respectively. Furthermore, the enzymes (e.g. flavonoid 3′-monooxygenase, bifunctional dihydroflavonol 4-reductase, anthocyanidin synthase, and so on) responsible for anthocyanin biosynthesis generate anthocyanin 3-O-glucosides that are significantly down-regulated. The down-regulated anthocyanin 3-O-glucosides were anthocyanin-3-O-rutinoside-5-O-glucoside and anthocyanin-3-O-(2''-O-glucosyl) glucoside, both of which were reduced by 2.16-fold. This reduction in content can be attributed to the semi-open fermentation environment, where in anthocyanins, when exposed to oxygen, undergo oxidative reactions due to the conjugated double bonds in their structure. Additionally, intermolecular polymerization can lead to cross-linking reactions between anthocyanin molecules (Ai et al., 2021).

3.3. Volatile compounds

Relevant studies have shown that the enrichment of volatiles in wine by non- *Saccharomyces cerevisiae* has been demonstrated several times (G. Zhang et al., 2018). Therefore, it is necessary to investigate the effect of it on the variation of volatile compounds in mulberry wine.

3.3.1. Multivariate statistical analysis

The cumulative contribution rates of the two principal components were 61.99 % and 12.94 % in PCA (Supplementary Fig. S5A). The results indicated the volatile compounds were effectively discriminated in samples (VSL, VSS, VMM, and VSL) from two stages of fermentation. Moreover, was not overfit, implying its strong fitting capacity. OPLS-DA analysis was also employed to discern the differing volatile compounds within different fermentation strategies (Supplementary Fig. 5B-E). OPLS-DA models shown $R^2Y > 0.99$, $Q^2 > 0.9$, cross-validation and permutation tests also suggest the reliability of the OPLS-DA model (Supplementary Fig. S6A-D). A total of 522 distinctive volatile compounds (VIP > 1, $P < 0.05$) were identified (Supplementary Fig. S10).

3.3.2. Differences in volatiles

The primary categories of volatile compounds were identified in S and M, encompassing esters, terpenes, organic heterocyclic compounds, aldehydes, etc. (Top 9) (Fig. 4). The types and relative abundance of these major volatile compound categories were observed in Fig. 4A and B, consistent with the results of hierarchical clustering analysis (HCA) (Supplementary Fig. S7). In M, substantial differences in the numbers of esters (82), terpenes (62), organic heterocyclic compounds (60),

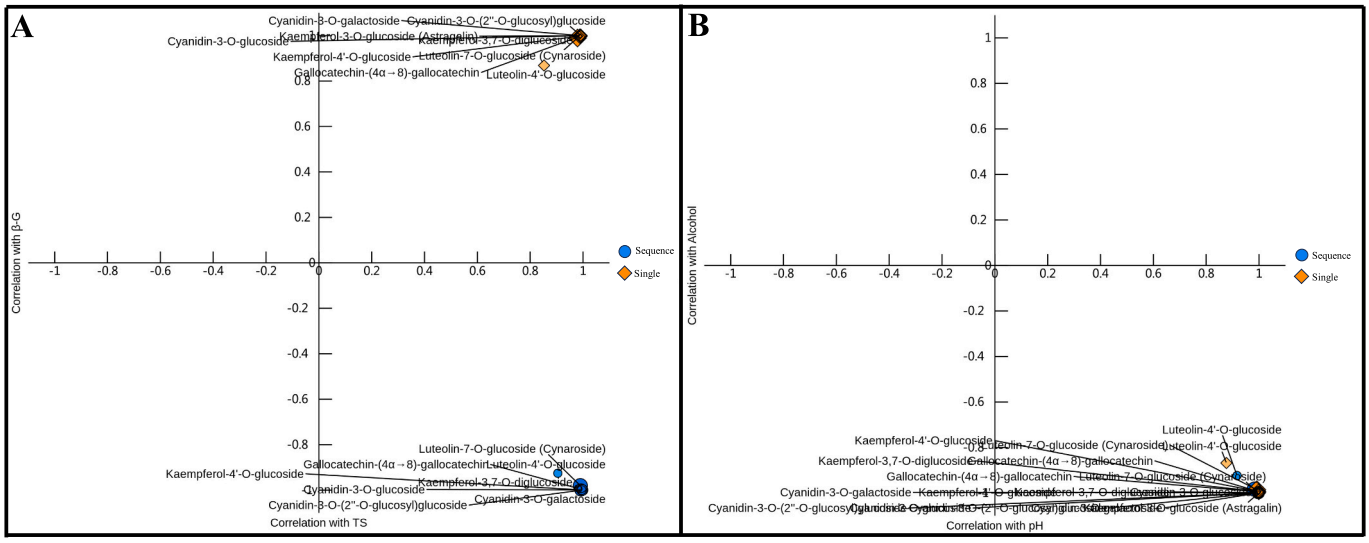

Fig. 2. Correlation of physicochemical parameters with major differential flavonoid under the different fermentation strategies (A: major differential flavonoid with β-G and TS correlation; B: major differential flavonoid with pH and Alcohol correlation).

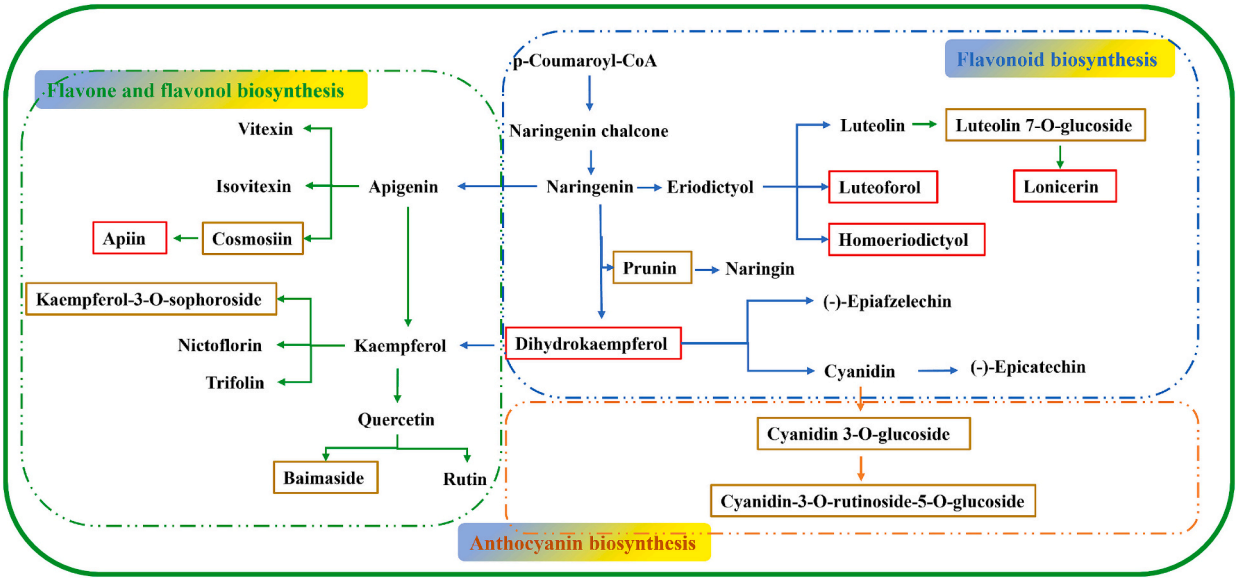

Fig. 3. The hypothetical metabolic pathway responsible for the enrichment of flavonoids is involved in M. For the whole, the compound frame in red represents up-regrated relative content, the compound frame in tawny represents the down-regrated relative content, while the substances without frames are the intermediate metabolites in the metabolic pathway. The green line and frame represent flavone and flavonol biosynthesis; the blue line and frame represent flavonoid biosynthesis; the yellow line and frame represent anthocyanin biosynthesis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hydrocarbons (22), alcohols (24), and aldehydes (28) were observed (exceeding 20), compared through the VMM (Volatile compounds-Single fermentation-Medium) vs. VSS (Volatile compounds-Single fermentation-Early) categories. For acids, ketones, and phenols, the differences in the number of categories were lower, with 13, 18, and 5, respectively. This suggests a stable growth in the types of volatile compounds during M fermentation, aligning with our expectations. Notably, remarkable differences in the types of volatiles were also observed between VSL (Volatile compounds-Single fermentation-Later) vs. VSS and VML (Volatile compounds-Sequential fermentation-Later) vs. VSS, with esters showing the most prominent disparity (exceeding 10). This was attributed to non-Saccharomyces yeast producing more distinct enzymes, such as esters and glycosidases, leading to an increase in the levels of ester categories. Additionally, the relative content of esters increased significantly by 2.38-fold compared to S. Among them, γ-Heptalactone, diethyl succinate, and butyric acid 1,1-dimethyl-2-phenylethyl ester were aroma compounds with relatively high content. Moreover, although terpenes, organic heterocyclic compounds, and aldehydes exhibited relatively small differences (⟨10) in the comparison between VSL vs. VSS and VML vs. VSS, their content levels were relatively high. Especially for organic heterocyclic compounds and terpenes. Furthermore, their relative content was significantly higher than that of S. Regarding organic heterocyclic compounds, including oxygen-containing (furan), sulfur-containing (thiophene), and ammonia-containing three heterocyclic compounds, increased significantly by 1.73-flod. It may also contribute to the aroma of caramel, fruits, and nuts in wine (Shi et al., 2024). For terpenes, the relative content of terpenes increased significantly by 1.8-fold. Among them, Zingiberene, m-

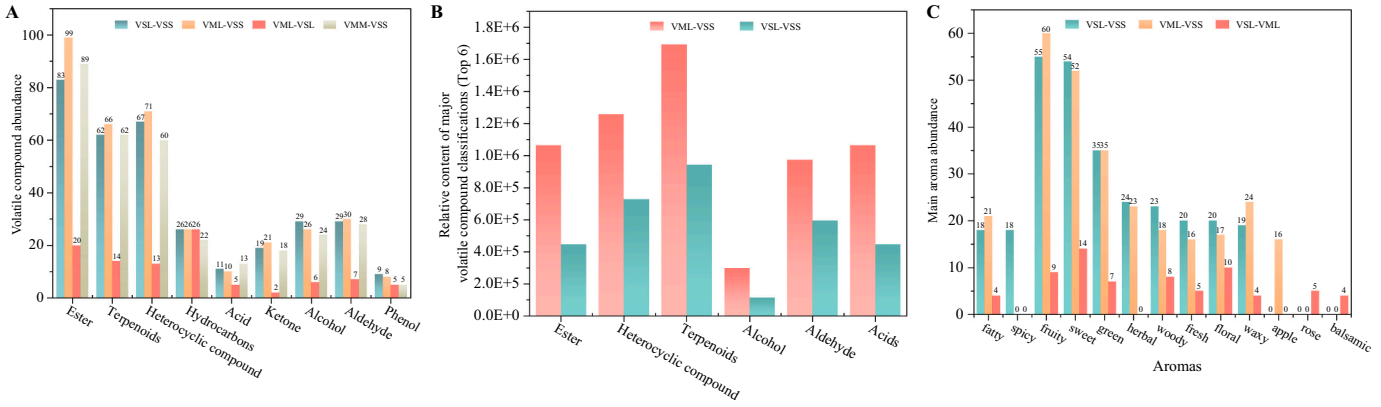

Fig. 4. Changes in volatile compound species and content in S and M (A: Differences in the abundance of main volatiles in different groups (VSL vs. VSS; VML vs.VSS; VML vs. VSL; VMM vs. VSS); B: Differences in the relative content of volatile compounds classified (Top 6) under the two fermentation strategies (VSL vs. VSS; VML vs.VSS); C: Abundance of compounds with aroma (Top 10) under different group (VSL vs. VSS; VML vs.VSS; VML vs. VSL).).

cymene, agarospirol, and nerolidol, cis-(+) were the top four most abundant terpenoids. Particularly, 8-hydroxy linalool, an important aroma compound derived from linalool, was screened for the first time in fermented mulberry wines, and its relative content increased significantly by 2.5-fold. Importantly, through the comparison of VML vs. VSL, within the main categories of volatile compounds, we observed an increase in the types of volatiles. In particular, there were significant differences in the categories of esters (20), terpenes (14), and organic heterocyclic compounds (13) (differences exceeding 10). Therefore, considering the differences in types and content levels between VSL vs. VSS and VML vs. VSS, along with the differences in categories between VML vs. VSL, the sequential fermentation strategy involving the addition of *M. pulcherrima* and *I. terricola* has the potential to enhance the production and growth of a substantial number of desired compounds. This strategy serves to facilitate the enrichment of volatile compounds in fermented mulberry wine, thereby exerting a potential influence on the aromatic characteristics of the mulberry wine.

To discern the intricate development of aroma in mulberry wine, the primary volatile compounds were categorized based on their distinctive aromatic profiles. A diverse range of aromas was evident (Top 10 of VSL vs. VSS, VML vs. VSS, and VML vs. VSL), including fruity, sweet, green, apple, rose, and more, as illustrated in Fig. 4C. Regardless of the fermentation strategy, sweet, fruity, and grassy notes were consistently prevalent among the aromatic compounds. In the case of the M fermentation strategy (VML vs. VSS), 16 aroma compounds (Fig. 4C), contributing to the apple flavor, were uniquely enriched, These compounds included 2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (*E*)-, 2-Buten-1-one, 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-, (E)-, Butanoic acid, 3-hexenyl ester, (*Z*)-, Butanoic acid, 3-methyl-, 1-methyl ethyl ester, and so on (Supplementary Fig. S8). In contrast, for the S fermentation strategy (VSL vs. VSS), 18 aroma compounds (Fig. 4C) contributing to a spicy flavor were uniquely enriched, including 2-furan propanoic acid ethyl ester, 2-heptanone, 2-octanol, and so on (Supplementary Fig. S8). It is noteworthy that these 16 compounds with an apple flavor and 18 compounds with a spicy flavor were significantly enriched in their respective fermentation strategies. The addition of non-*S. cerevisiae* may promote the production of compounds with apple flavor characteristics and suppress the production of compounds with a spicy flavor. Furthermore, through VML vs. VSL, it was observed that in the late stage of fermentation, M exhibited a greater enrichment of aroma compounds compared to S. The compounds contributed to a richer sweetness (14), fruity notes (9), and floral aroma (10) in M (Fig. 4D). Notably, 5 rose (e.g. benzyl alcohol, ethyl geranate, hydrocinnamic acid) and 4 balsamic ((−)-myrtenol, benzoic acid 1-methyl-ethyl ester, benzyl alcohol, p-cymen-7-ol) aroma were categories of the significant present in VML vs. VSL (Fig. 4D, Supplementary Fig. S8). Consequently, upon comparing the aroma profiles of groups M and S, it was evident that the aromatic spectrum in M is notably more abundant and diverse. In contrast to similar studies on sequential fermentation, the sequential fermentation involving both *M. pulcherrima* and *I. terricola* demonstrates a substantial enhancement in the overall acceptability and aromatic complexity of the mulberry wine.

3.3.3. Correlation between major volatile compounds and physicochemical properties

For volatile compounds, alcohol content, β-glucosidase activity, and total sugar show a significant positive correlation with the group of volatile compounds in the single-strain fermentation strategy ($P < 0.05$, Fig. 5A and B). This indicates that during alcohol fermentation, *S. cerevisiae* may actively regulate the production of aromatic volatile compounds by utilizing the alcohol and active β-glucosidase generated from the abundant sugars (P. Zhang et al., 2021). In contrast, in sequential fermentation, due to the distinct metabolic preferences, priorities, and substrate utilization characteristics of the inoculated microorganisms (*M. pulcherrima* and *I. terricola*), the pathways could lead to alcohol production may have a higher demand for sugars (Koh et al., 2024) (Duncan et al., 2023). This could potentially reduce the availability of precursors for aromatic volatile compounds (Maoz et al., 2022), resulting in a negative correlation between alcohol content and β-glucosidase activity with aromatic volatile compounds ($P < 0.05$, Fig. 5A and B). Additionally, the non-*Saccharomyces cerevisiae* (*M. pulcherrima* and *I. terricola*) demonstrate high sugar utilization efficiency and strong adaptability to the environment (Steensels et al., 2014). The complex interactions with other microorganisms could lead to a significant positive correlation between total sugar and pH with aroma compounds ($P < 0.05$, Fig. 5A and B).

3.3.4. Volatile compounds metabolism pathways analysis

Similar to the enrichment pattern of flavonoid compounds, both S and M groups exhibit overlap in the KEGG metabolic pathways enriched with volatile compounds (Supplementary Fig. S9A and B), the top 20 metabolic pathways in each group were enriched (Supplementary Fig. S9A and B), primarily centered around arginine and proline metabolism, butanoate metabolism, and 2-oxocarboxylic metabolism. It is noteworthy that the fermentation process in the M group may potentially further enhance the metabolic processes of tryptophan metabolism (www.genome.jp\dbget-bin\www_bget?map00380), resulting in a greater accumulation of metabolites in this pathway compared to the S group. In addition, Indole alkaloid biosynthesis (www.genome.jp\dbget-bin\www_bget?map00901) was also uniquely enriched in M (Supplementary Fig. S9A and B).

For aroma compounds following sequential inoculation with

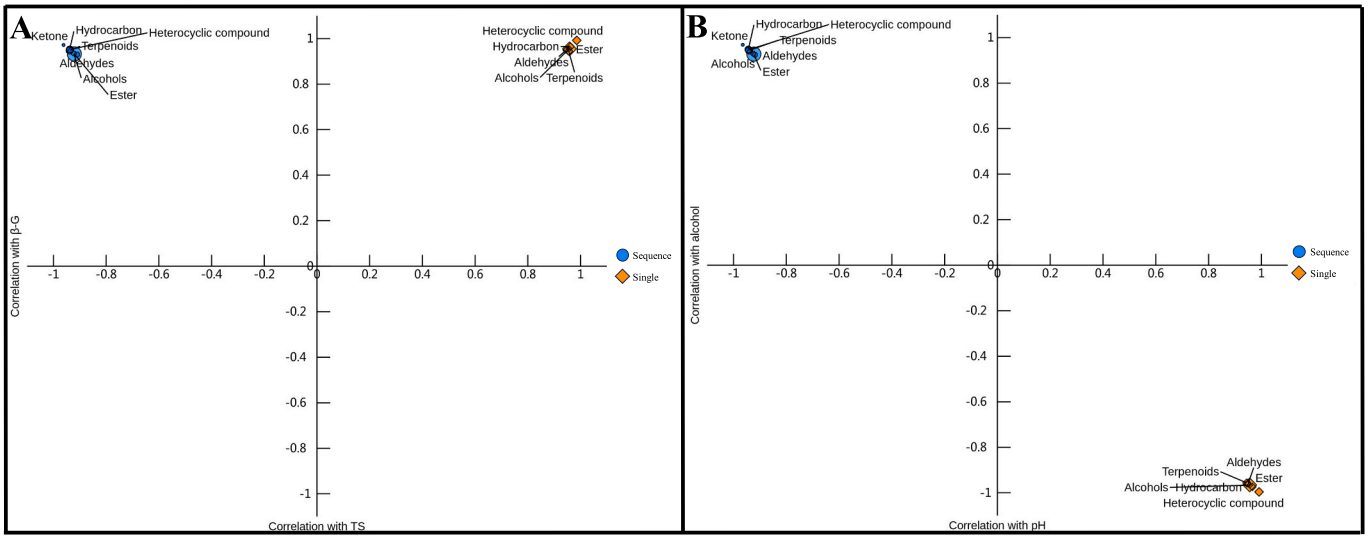

Fig. 5. Correlation of physicochemical parameters with the main classification of volatiles under the different fermentation strategies (A: main classification of volatiles with β-G and TS correlation; B: main classification of volatiles with pH and Alcohol correlation.).

M. pulcherrima and *I. terricola*, we analyzed the VML vs. VSS. As depicted in Fig. 6, the enriched aroma compounds predominantly result from the catabolism of aromatic amino acids via biosynthesis was hypothecated by our study. Butanal, 3-methyl-, oxime (significantly up-regulated by 3.02-fold), Butanoic acid (significantly up-regulated by 4.82-fold, imparting "Sour" and "Rancid" notes), Hydrocinnamic Acid (significantly up-regulated by 1.29-fold, contributing a "Cheesy" aroma), trans-sinapyl alcohol (significantly up-regulated by 16.40-fold), and phenol, 2-methoxy-4-(1-propenyl)- (significantly up-regulated by 1.68-fold, offering "Sweet," "Woody," and "Floral" characteristics) were enriched in our analysis (Supplementary Fig. S10), as indicated by the KEGG pathway. These compounds were largely the result of the collaborative action of microbial entities (e.g., yeast, molds) and plant cell-produced enzymes, including aminotransferases, ammonia-lyases, mutases, dehydrogenases, reductases, transferases, and synthases (Steensels et al., 2014) (Skaliter et al., 2022). Crucially, Tryptophan metabolism and Indole alkaloid biosynthesis emerge as uniquely enriched metabolic pathways under the sequential fermentation strategy. Here, we hypothecated tryptophol, imbued with a rose-like aroma (significantly up-regulated by 9.71-fold), the earthy-scented Indole, 3-methyl- (significantly up-regulated by 8.39-fold), and 9H-Pyrido[3,4-*b*]indole, 1-methyl- (significantly up-regulated by 10.85-fold) were generated by chorismate substrate catalysis through pyruvate decarboxylation, employing a multitude of enzymes (e.g. tryptophan transaminase, aromatic-L-amino acid/tryptophan decarboxylase, and so on) (Fig. 6).

4. Conclusion

In this study, β-glucosidase activity was greater and fermentation time was declined to 144 h. 11 flavonoid metabolites are significantly

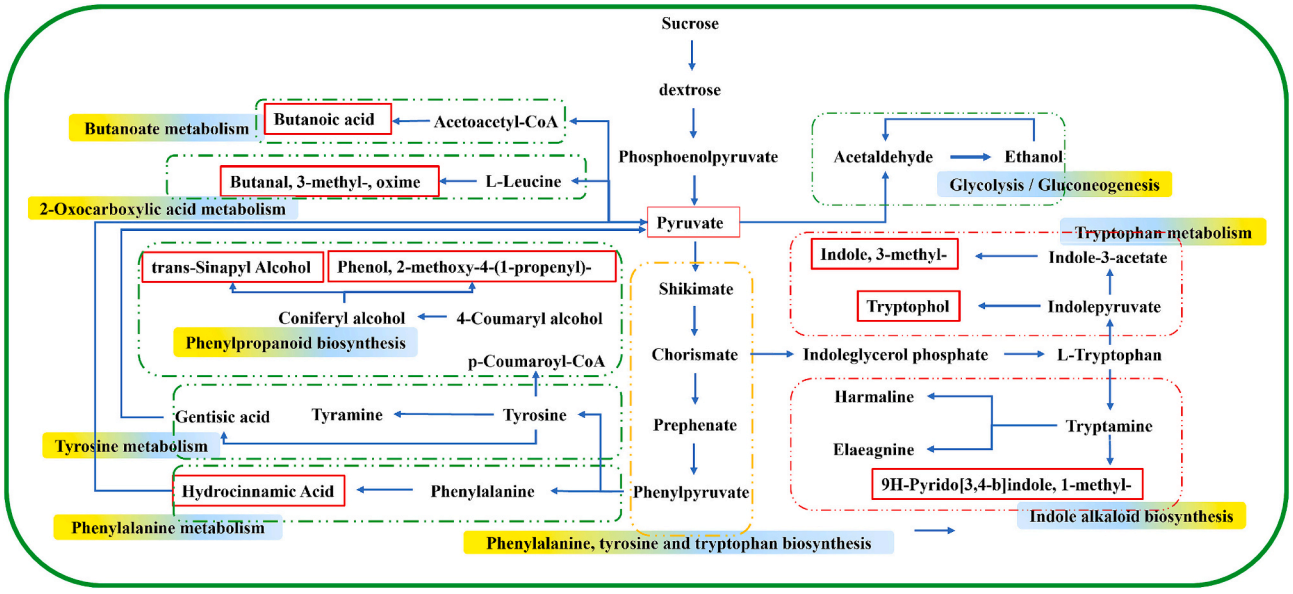

Fig. 6. The hypothetical metabolic pathway responsible for the enrichment of volatiles is involved in M. For the whole, the compounds frame in red represents up-regrated relative content, the compounds frame in tawny represents the down-regrated relative content, while the substances without frames are the intermediate metabolites in the metabolic pathway. Red frames represent metabolic pathways uniquely involved in M; The green frames represent metabolic pathways where M and S overlap; the yellow frames represent intermediate metabolic pathways important in M. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

elevated. Furthermore, metabolites enriched in the biosynthesis of flavonoids and flavonols, as well as anthocyanin biosynthesis, accounted for 46.15 % and 23.08 %, respectively. Correlation analyses with physicochemical parameters indicate a significant positive correlation ($P < 0.05$) between β-glucosidase activity, alcohol, and flavonoid content. Regarding volatile compounds, the types and contents of major compound classes are greater, especially esters, terpenes, and heterocyclic compounds. Moreover, 16 aroma compounds, contributing to the apple flavor, were uniquely enriched. Rose (5) and balsamic aroma (4) were categories distinctly present in the late stage of fermentation. A significant positive correlation was obverted between total sugars, pH, and aroma compounds. Importantly, the identification of Indole, 3-methyl-, Tryptophol, and 9H-Pyrido[3,4-*b*]indole, 1-methyl-, suggests their synthesis through the uniquely present pathways of Tryptophan metabolism and Indole alkaloid biosynthesis. The comparative analysis of different fermentation strategies aids in comprehending the value of introducing *Metschnikowia pulcherrima* and *Issatchenkia terricola* as sequential co-fermenters in enhancing the sensory and nutritional quality of mulberry wine, thereby contributing to the optimization of the fermentation process. This study offers a robust framework of recommendations and scientific directives for optimising the quality of mulberry wine.

CRediT authorship contribution statement

Haotian Xu: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Zeyu Wang:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Zhenyang Qin:** Data curation. **Minwei Zhang:** Writing – review & editing. **Yanan Qin:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101869.

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