Comparative metabolic profiling of Cabernet Sauvignon wines reveals the potential of different *Wickerhamomyces anomalus* co-fermented with commercial *Saccharomyces cerevisiae*

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**ABSTRACT**

In this paper, five *Wickerhamomyces anomalus* accumulated 9.1–19.7% less released CO₂ than *S. cerevisiae* in the synthetic must. Untargeted metabolomics was used to analyze co-fermented real Cabernet Sauvignon wines. The results showed that all *W. anomalus* strains significantly decreased the levels of malic acid and hypoxanthine in co-fermented wines, thereby promoting the process of TCA cycle and purine metabolism, respectively. Furthermore, WG5, WG26, and WG27 strains utilized more amino acid, thus favoring the enhancement of aroma compounds. WG27 strain also could biotransform more odorless metabolites into flavor active compounds, such as phenethyl alcohol, indole and indole-3-acetaldehyde. Notably, WG27 strain significantly up-regulated secondary metabolites by more than 400% compared to the other *W. anomalus* strains. These secondary metabolites were involved in phenylpropanoid and flavonoid metabolism, tryptophan metabolism and methionine metabolism. In addition, co-fermentation with the WG27 strain also resulted in an increase in antioxidant activities by more than 16.09% compared to the control wine. This suggests that the WG27 strain not only improves the flavor and style of the wine but also enhances its antioxidant properties. The findings can be helpful in expanding general understanding of *W. anomalus* and its potential applications in the food and beverage industry.

1. Introduction

In winemaking, *Saccharomyces cerevisiae* dominate over non-*Saccharomyces* during alcohol fermentation, but enologist are increasingly interested in the beneficial role of non-*Saccharomyces* yeasts, such as improving the acidity, aromatic complexity, ethanol reduction and polysaccharide concentrations, producing bioactive compounds, and so on (Beatriz et al., 2016; Ellis et al., 2022; Mateo & Maicas, 2016; Padilla et al., 2018; Álvarez-Fernández et al., 2020). These growing interest in developing the complexity and diversity of wines to meet the market demand has led to the constant search for new strains of yeast to create unique varieties of wine. Numerous yeast strains including *Hanseniaspora uvarum*, *Issatchenkia orientalis*, *Lachancea thermotolerans*, *Metchnikowia pulcherrima*, *Pichia kluyveri*, *Torulaspora delbrueckii*, and more have proven so positive effect so that some strains have been commercialized to improve wine quality (Benito et al., 2019; Padilla et al., 2018). *Wickerhamomyces anomalus*, which can be found on grapes in the vineyard and wine, has the potential to change chemical constituents and enhance aroma complexity, such as acetate and ethyl esters, monoterpenes, higher alcohols, and acids (Lee & Park, 2020; Padilla et al., 2018). We previously reported on the potential of three *W. anomalus* strains for co-fermentation of Cabernet Sauvignon wines to enhance wine quality and enrich wine styles by investigating physiological and enological characterizations (Wang et al., 2023).

However, there is still limited information available regarding the deeper metabolism of *W. anomalus* strains during co-fermentation with *S. cerevisiae*. Furthermore, we do not yet understand the response of *W. anomalus* strains to the stress of fermentation environment. This knowledge is necessary from the industry’s perspective to develop better fermentation strategies that consider the adaptive behavior of *W. anomalus* strains during co-fermentation with *S. cerevisiae*. These growing interest in developing the complexity and diversity of wines to meet the market demand has led to the constant search for new strains of yeast to create unique varieties of wine. Numerous yeast strains including *Hanseniaspora uvarum*, *Issatchenkia orientalis*, *Lachancea thermotolerans*, *Metchnikowia pulcherrima*, *Pichia kluyveri*, *Torulaspora delbrueckii*, and more have proven so positive effect so that some strains have been commercialized to improve wine quality (Benito et al., 2019; Padilla et al., 2018). *Wickerhamomyces anomalus*, which can be found on grapes in the*

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sensitivity, and can be used for accurate qualitative and non-directional unknown screening in samples (Wu et al., 2022). Recently, an increasing number of untargeted wine UHPLC-Q-TOF-MS metabolomics has appeared in the literature (Arapitas & Perenziöni et al., 2012; Arapitas et al., 2014; Ontanon et al., 2020; Pan et al., 2023). These studies offer a new and global perspective on various oenological issues and provide new hypotheses for further studies. With respect to non-Saccharomyces yeasts, Minebois (Minebois et al., 2020a; Minebois et al., 2020b) and López-Malo (López-Malo et al., 2013) compared metabolism of S. cerevisiae, S. uvarum and S. kudriavzevii species in wine fermentation processes or under low temperature conditions. Álvarez-Fernández (Álvarez-Fernández et al., 2019; Álvarez-Fernández et al., 2020) reported on aromatic amino acids metabolism using T. delbrueckii in sequentially inoculated fermentations with S. cerevisiae. Previously, the literature reported on the ethanol stress response metabolomic mechanism in W. anomalus strains (Y. Li, Long, et al., 2022). However, understandable metabolic profiles during W. anomalus co-fermentation with S. cerevisiae have not yet been determined. Specifically, the metabolomic fingerprint of different W. anomalus strains producing various wine styles in co-fermentation is still unknown.

Moreover, our research has revealed that different W. anomalus strains have varying effects on the levels of polyphenolic compounds (Wang et al., 2023). Indeed, many studies have been conducted to show that polyphenol content in wine is correlated to its antioxidant properties and these exogenous antioxidants can as reactive oxygen species-scavengers to enhance human health (Li et al., 2009). However, the effect of W. anomalus on secondary metabolites with antioxidant activity in co-fermenting red wines is not well-established. The aim of this study was to use a high throughput untargeted UHPLC-Q-TOF-MS method to create a comprehensive and untargeted overview of the metabolites changes induced by the addition of different W. anomalus strains in co-fermentation with S. cerevisiae. Our study anticipates providing original insights into the metabolomic fingerprint of different W. anomalus strains adapted to the fermentation environment and to reveal the metabolic mechanisms responsible for the elevated antioxidant activity.

2. Materials and methods

2.1. Yeast strains and growth conditions

The five W. anomalus strains (WG5, WG6, WG11, WG26 or WG27) were isolated earlier from wine grape cultivated in vineyards located at the National Grape Variety Resource Nursery (Taigu, Shanxi) and was identified by sequence analysis of the 26S rRNA D1/D2 domain (Wang et al., 2023). The S. cerevisiae used was EXCELLENCE® XR (Lamothe Abiet; Canéjan, France). All W. anomalus strains were preserved in the laboratory and were maintained at −80 °C prior to use. Yeasts were routinely grown at 25 ± 2 °C on YPD and synthetic must (adjusted pH 3.0) that simulated standard grape juice was as follows: glucose (100 g/L), fructose (100 g/L), yeast extracts (1.5 g/L), citric acid (0.3 g/L), malic acid (5 g/L), tartaric acid (5 g/L), (NH4)2SO4 (2 g/L), MgSO4 (0.4 g/L), KH2PO4 (5 g/L), NaCl (0.2 g/L), MnSO4 (0.05 g/L) (Hu et al., 2018). Static culture and fermentation were carried out in 1 L Erlenmeyer flasks with 750 mL of synthetic must in which total inoculation amount was controlled at 10⁶ cells/mL for each yeast. Co-culture by each 10⁶ cells/mL of W. anomalus strains with 10⁶ cells/mL of S. cerevisiae were simultaneously inoculated and noted as follows: SWG5, SWG6, SWG11, SWG26, and SWG27. Growth was monitored spectrophotometrically by measuring the optical density (OD600) and fermentation progress was daily monitored by weight loss (indicative of CO2 generation). The ethanol content, titratable acidity, volatile acidity, and reducing sugars were determined using official methods (OIV, 2011).

2.2. Wine fermentation

The Cabernet Sauvignon was harvested from a commercial vineyard (vintage 2020) located in Tai Gu, Shan Xi, China (37.42N, 112.53E). The winemaking followed the procedures described in our earlier literature (Wang et al., 2023). The grape berries were destemmed, crushed, and pumped to 25 L stainless steel tanks, followed by the addition of 50 mg/L sulfur dioxide (SO2) and 20 mg/L of pectolytic enzymes (Novozymes, Tianjin, China) with an interval of 4 h. The W. anomalus and S. cerevisiae were inoculated with 10⁶ CFU/mL respectively and the co-fermentation was initiated by simultaneous inoculation and was marked as above. The traditional vinification for mono-fermentation was started by the addition of 2 × 10⁶ CFU/mL of W. anomalus or S. cerevisiae (noted as Sc) used as control. Alcoholic fermentation was maintained at 25 ± 2 °C until the alcoholic fermentation was completed when the residual sugar concentrations were below 2 g/L. Then, wines were settled for 48 h at −4 °C prior to racking off the lees with the addition of 50 mg/L SO2. After continuing cold stabilization for 1 month, metabolomics and antioxidant activity analyses were done.

2.3. Analysis of antioxidant activity

The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate), ABTS (2,2’-azinobis- (3-ethylbenzthiazoline-6-sulphonate)) and FRAP (ferric reducing-antioxidant power) assays were measured according to the method published previously (Lingua et al., 2016). Results were expressed as trolox equivalent antioxidant capacity. Trolox standard solutions were prepared at a concentration ranging from 4 to 120 mM.

2.4. UHPLC-Q-TOF-MS analysis

Wines were directly filtered with 0.2 μm PTFE filters into a 2 mL amber vial (MS certificated) prior to LC-MS analysis. An Agilent UHPLC-Q-TOF-MS 6545 system was equipped with an Eclipse Plus C18 chromatographic column (150 mm × 3.0 mm, 1.8 μm; Agilent, United States), following the procedures described in the literature (Yan et al., 2022 a; b). The mobile phase A and B were 5 mM ammonium formate in water and methanol respectively, and 0.1% formic acid was used as additive in both eluents. A gradient elution program ran with as follows: 0–1 min, 99% A; 1–5 min, 85% A; 5–25min, 1% A; 25–30 min, 1% A; 30–31 min, 99% A; and 31–35 min, 1% A. Each injection of 2 μL was loaded with a flow rate of 0.3 mL/min. The optimized MS parameters were set as follows: drying gas temperature was 250 °C with a flow rate of 11 L/min, sheath gas temperature was 300 °C with a flow rate of 12 L/min, nebulizer pressure was 35 psi, fragmentor voltage was 135V, VCap was 3000 V, collision energy was 20 V and mass ranges was 65–1700 mz. Mass accuracy was calibrated using reference ions 121.0508 and 922.0098 for positive mode, 112.9858 and 1033.988109 for negative, respectively.

2.5. Data processing

The Mass spectrum data were processed using Agilent MassHunter version B.10.00. The molecular features (use peaks with height ≥1000 counts, a 0.2 min of retention time error, 2 mDa of relative error for mass) were extracted by Profinder 1.0 software and obtained data were saved in CEI format.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) of the data and the determination of the significance of the difference were performed using SPSS 21.0 software. The least significant difference test was used to measure variations between treatments at a probability level of p < 0.05. In terms of metabolomic data, univariate statistical analysis, multivariate statistical analysis and compound identification were performed in Mass...
Profiler Professional (MPP, Agilent Technologies software). Furthermore, pathway analyses were performed using Metaboanalyst 4.0.

3. Results

3.1. Growth and fermentative characteristics of five *W. anomalus*

In synthetic must, WG6 and WG27 strains exhibited lower growth than other *W. anomalus* during the initial logarithmic period. This lower growth persisted even after entering the stability period (Fig. 1A). Furthermore, we also observed that WG6 strain had the lowest accumulation of released CO₂ during fermentation. The amount of CO₂ released per day in the mid-period of fermentation was lower than that of the other *W. anomalus* and maintained at 3.6–3.9 g/L (Fig. 1B and C). In comparison, the fermentation rate (CO₂ released per day) of all *W. anomalus* was lower than that of *S. cerevisiae* during the middle stage of fermentation from the fifth to the tenth day. Hence, at the end of fermentation process in synthetic must, all *W. anomalus* accumulated 9.1–19.7% less released CO₂ than *S. cerevisiae* (Fig. 1C). During co-fermentation, the fermentation rates of both *W. anomalus* and *S. cerevisiae* were not affected at the beginning of the fermentation because amount of CO₂ released per day was twice as much as in the respective individual yeast fermentations with the same inoculum (Fig. 1B). However, as the fermentation proceeds beyond the eighth day, CO₂ release gradually decreased, tending to coincide with that of the *S. cerevisiae*, indicating that *S. cerevisiae* was dominant at this time. In addition, we measured the enological parameters on the eighth day and the results showed that all *W. anomalus* strains had lower sugar utilization and ethanol content than that of *S. cerevisiae*. These findings were consistent with the lower accumulation of CO₂ release by *W. anomalus* strains. Additionally, the higher titratable acidity and volatile acidity were produced in co-fermented wines than Sc wine (Supplementary Table S1).

3.2. Metabolomic profiling of different co-fermented wines

In Cabernet Sauvignon wines, a PCA model (ESI+ and ESI-) in unsupervised mode was applied to the metabolites of samples and QC samples were closely clustered, supporting the high quality of the experiment (Fig. 2). We observed that the separation between all co-fermented wines and the control (Sc) wine was clear in both ESI+ and ESI- PCA plots. Furthermore, the SWG11 wine samples were located away from the other wine samples, as were the SWG27 wines, suggesting that these two wines had big differences from the others. The SWG5, SWG6 and SWG26 wines were confused together and not clearly distinguished from each other. Hence, all co-fermented wines were distributed within three different regions.

3.2.1. Determination of differential markers

Furthermore, we performed univariate statistical analysis, including t-test (*p* value) and fold-changes (FC), between each type of co-fermentation and *S. cerevisiae* fermentation alone (Ahn et al., 2020). Differential metabolites (including unidentified substitutes) with a *p*-value < 0.05 and FC > 2 (or < 0.5) was selected and five Volcano Plot showing log 2-fold-changes against minus log 10 *p*-values visually had a similar distribution (Supplementary Fig. S1). As shown in Fig. S1 A–J (ESI+ and ESI-), the differences between the SWG27 wine and the control wine samples were the most obvious and had the most differential metabolites. The OPLS-DA score plots, based on these differential metabolites, showed a high degree of discrimination between each type of co-fermentation and *S. cerevisiae* fermentation alone, with a clear separation between the fermentation groups (Supplementary Fig. S2). In OPLS-DA analysis, we examined the differential metabolites, which had satisfied *p*-value < 0.05 and FC > 2 (or < 0.5), of VIP value > 1 to identify potential biomarkers. These selected metabolites were further identified by molecular weight in the tandem mass spectrometry database.

Fig. 1. Growth of five *W. anomalus* strains in the synthetic must (A). Amount of CO₂ released per day (B) and end of process (C) during fermentation of synthetic must using different *W. anomalus* alone and co-fermentation with *S. cerevisiae*.
Fig. 2. PCA score plots of samples for co-fermentation of different strains of *W. anomalus* with *S. cerevisiae*.

Fig. 3. Summary of identified metabolites by comparing of various co-fermentation vs *S. cerevisiae* fermentation alone. (A) Bar graph displaying the number of up-regulated and down-regulated differential metabolites detected in positive and negative ion modes. (B) Venn diagram displaying relations of differential metabolites.
(METLIN), HMDB, KEGG and TCM database.

After comparing with \textit{S. cerevisiae} fermentation alone, 165 differential metabolites (48 in ESI- and 118 in ESI+, with 1 present in both modes) in SWG5 wine, 125 metabolites (46 in ESI- and 79 in ESI+) in SWG6 wine, 111 metabolites (35 in ESI- and 76 in ESI+) in SWG11 wine, 142 metabolites (56 in ESI- and 86 in ESI+) in SWG26 wine and 543 metabolites (287 in ESI- and 290 in ESI+, with 34 present in both modes) in SWG27 wine were annotated as different types of compounds (Supplementary Tables S2–S3). The identified metabolites include carbohydrates, organic acids, fatty acids, amino acids, vitamins, nucleotides, phenols, alcohols, esters, alkaloids, glucosinolates and other metabolites. As shown in Fig. 3A, ESI- had more up-regulated differential metabolites than down-regulated ones. Conversely, ESI+ had an increased proportion of down-regulated differential metabolites. These indicated that organoalkali compounds (such as alkaloids) may be more down-regulated based on the common occurrence in ESI+. Furthermore, the KEGG enrichment of differential metabolites was analyzed. Compared with \textit{S. cerevisiae} fermentation alone, co-fermentation mainly enhanced the phenylpropanoid biosynthesis and metabolism, flavonoid biosynthesis and metabolism, biosynthesis and metabolism of amino acids, aminocyt-rRNA biosynthesis, tropane and piperidine alkaloid biosynthesis, aromatic polyketides and stilbenoid biosynthesis, terpenoid backbone and steroid biosynthesis and metabolism, fatty acid biosynthesis and metabolism, vitamin B6 metabolism, purine metabolism, and so on (Fig. 4).

3.2.2. Metabolomes comparison with \textit{S. cerevisiae} fermentation alone

The Venn plot displayed the intersection analysis that revealed only fourteen differential metabolites as common in all co-fermentation samples in comparison to the control group (Fig. 3B). Only a few metabolites, namely malic acid, hypoxanthine, hydroxytyrosol, and fraxetin, and methylantranilate, exhibited enrichment into the KEGG pathways. Furthermore, among of all enriched KEGG pathways, only two metabolic pathways were altered in each of co-fermentation

Fig. 4. KEGG pathway enrichment analysis (A: SWG5/Sc; B: SWG6/Sc; C: SWG11/Sc; D: SWG26/Sc; E: SWG27/Sc).
compared to controls (Fig. 4). They were phenylalanine, tyrosine and tryptophan biosynthesis, and purine metabolism. Subsequently, further analysis mainly focused on comparison of the impact of W. anomalus strains on primary and secondary metabolism of co-fermentation.

For primary metabolism, first of all, tricarboxylic acid (TCA) cycle plays a key role in central carbon metabolism. Significant decrease of malic acid content in all co-fermented wines was observed, suggesting a positive impact of co-fermentation on the TCA cycle and its ability to enhance energy and material metabolism. In the central carbon metabolism of SWG27/Sc, levels of some polyols and organic acids, such as pyruvate, malic acid, tartaric acid, mannitol, and erythritol dramatically declined, while isocitric acid level increased (Fig. 5). Furthermore, the intermediate metabolites in pyruvate metabolism, such as mesaconic acid and methylglyoxal were up-regulated in SWG27/Sc. Secondly, the tryptophan biosynthesis, and purine metabolism. Subsequently, further aromatization of amino acids (shikimate pathway links carbohydrates metabolism to biosynthesis of aromatic amino acids (l-tryptophan, l-phenylalanine, and l-tyrosine) and many aromatic secondary metabolites (Herrmann & Weaver, 1999). With the exception of SWG5 wine, shikimic acid levels were lower in all the co-fermented wines than in the control wine. Furthermore, the content of l-phenylalanine decreased in SWG5/Sc and SWG26/Sc but increase in SWG6/Sc (Fig. 5). Regarding non-aromatic amino acids, WG6 strain also increased the levels of l-methionine, l-leucine, alloiso-leucine and l-histidine in SWG6 wine, whereas the levels of one or more non-aromatic amino acids (including l-lysine, lysopine, l-homoserine, l-pyroglutamic acid, l-theanine and l-ornithine) decreased in other co-fermented wines. Thirdly, with regard to fatty acids, both up-regulated and down-regulated differential metabolites were prevalent in SWG26/Sc and SWG27/Sc, indicating that the biosynthesis and metabolism of fatty acids in WG26 and WG27 strains were very active. Especially with SWG27 wine, levels of more than 50% fatty acids (including caprylic acid, isovaleric acid, sebacic acid, trichosanic acid, tiglic acid and coronaric acid) increased while several others (including isobutyric acid, enanthic acid and dimeric acid) decreased. The variability of up-regulated differential metabolites of fatty acids in SWG27/Sc and amino acids in SWG6/Sc helped us to understand the lower cell biomass and fermentation weight loss in these strains than in the other three strains as described in the aforementioned experiments, whether in solo or co-culture with S. cerevisiae. Finally, the level of hypoxanthine was decreased in any of W. anomalus strain co-fermented wines. Moreover, Vitamin B6 and Vitamin B12 levels experienced a decline in SWG27/Sc and SWG6/Sc, respectively, while Vitamin B9 was lower in SWG5 and SWG27 wines than in the Sc wine.

In terms of secondary metabolism, the primary differential compounds were the downstream of shikimate pathway (Table 1 and Supplementary Table S4). Regarding the phenylpropanoid branch of shikimate pathway, phenethylamine and phenethyl alcohol levels increased in SWG27 wine, along with nine cinamic acids and derivatives by phenylpropanoid biosynthesis pathway up-regulated in SWG27/Sc. In contrast, WG5 and WG6 strains only increased one phenylpropanoid compound, respectively. As a result of the downstream pathway of phenylpropanoid metabolism, the number of up regulated flavonoid, coumarins, aromatic polyketides and stilbenoid flavonoids and polyphenols in SWG27 wine was fourfold higher than in the other co-fermented wines. We drew a metabolic pathway map for SWG27 wine to better visualize the changes in the different metabolites and metabolic pathways (Fig. 6). Furthermore, hydroxytyrosol, a product of tyrosine metabolism, was significantly reduced in all co-fermented wines, while two indole and derivatives produced by the tryptophan metabolic pathway were exclusively up-regulated in SWG27/Sc. Glucosinolates biosynthesis via methionine metabolism and terpenoid backbone and steroid biosynthesis were activated in SWG5 and SWG27 wines, particularly in SWG27 wine where four glucosinolates and six terpenoid and steroid metabolites were up-regulated. Methylanthranilate levels were increased by all W. anomalus strains in co-fermented wines. Although, the up- and down-regulation of various alkaloids was not uniform in all the co-fermented wines, SWG27 wine had down-regulated alkaloids instead of up-regulated ones as was observed in SWG6 wine.

3.3. Antioxidant capacity of different co-fermented wines

Based on the up-regulated of some flavonoids and other secondary metabolites, we evaluated the antioxidant activity of all wine samples, including DPPH, ABTS and FRAP assays. The three antioxidant activity assays exhibited consistent and significant elevation in SWG5, SWG6 and SWG27 wines relative to the control wine samples (p < 0.05), except for FRAP value in SWG5 and SWG6 wines (Table 2). In particular, SWG27 wine exhibited the highest antioxidant activities, including an increase of 16.09% in DPPH value, 80.31% in ABTS value and 22.39% in FRAP value. The observed results aligned with the highest up-regulation of certain secondary metabolites in SWG27 wine, indicating that the improvements in antioxidant activity of the wines were a consequence of the elevated levels of these secondary metabolites.

4. Discussion

4.1. Effect of co-fermentation on primary metabolism

4.1.1. Carbohydrates and organic acids

Although co-fermentation resulted in faster sugar consumption than fermentation by S. cerevisiae alone in synthetic must, the final sugar levels did not differ significantly between co-fermented and control
wines, as determined by UHPLC-Q-TOF-MS analysis. In addition, co-fermented wines had lower levels of malic acid, an organic acid produced during the fermentation process when sugars enter the TCA cycle through glycolysis. This degradation of malic acid is attributed to the ability of *W. anomalus* strains to more efficiently utilize this compound and utilize it as a sole source of carbon (Satora et al., 1990; Saayman & Viljoen-Bloom, 2006). Our studies showed a similar trend to previous studies by Satora (Satora et al., 2014) and Sun (Sun et al., 2022) on apple and kiwi wines, respectively. Furthermore, metabolites such as mannitol, erythritol and tartaric acid were also observed at lower levels in SWG27 wine. This decrease was consistent with reports of some *W. anomalus* strains that can assimilate these polyols, but *S. cerevisiae* and others *W. anomalus* strains cannot (Kim et al., 2013; Zaky et al., 2016). Previous research has stated that non-*Saccharomyces* can degrade more tartaric acid than *S. cerevisiae* in the sequential inoculation (Dutraive et al., 2019). Consistent with these reports, WG27 strain could utilize these specific polyols and use more tartaric acid for its metabolisms. Meanwhile, the SWG27 wine had lower levels of quinic acid and shikimic acid, intermediate metabolites of the shikimate pathway that supplies precursors to phenolic compounds such as phenolic acids and flavonoids (Herrmann & Weaver, 1999; Minebois et al., 2020a). These decrease indicated an intensification of this metabolic branch by WG27 strain. Our hypothesized that metabolites underwent conversion towards phenylalanine metabolism, and subsequently towards phenylpropanoid biosynthesis, was supported by the up-regulation of products such as phenethyl alcohol, flavonoid derivatives, and cinnamic acid derivatives. Zaky (Zaky et al., 2016) also reported that a strain of *W. anomalus* can utilize quinic acid, which was consistent with our findings on WG27 strain. The lower levels of

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Table 1
Quantities of major secondary differential metabolites in metabolic pathway of different co-fermented Cabernet Sauvignon wines.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Number of up-regulated compounds</th>
<th>Number of down-regulated compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWG5</td>
<td>SWG6</td>
<td>SWG11</td>
</tr>
<tr>
<td>Biosynthesis of alkaloids derived from shikimate pathway(^a)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Phenylalanine metabolism</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Phenylpropanoid biosynthesis</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Flavonoid, coumarin, aromatic polyketides and stilbenoid biosynthesis</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Tryptophan metabolism</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoid backbone and steroid biosynthesis and metabolism</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Methionine metabolism and glutinosolates biosynthesis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tyrosine metabolism(^b)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Biosynthesis of various alkaloids</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\) The compound up-regulated in this pathway for all co-fermented wine samples was methylanthranilate.

\(^b\) The compound down-regulated in this pathway for all co-fermented wine samples was hydroxytyrosol.

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### Table 2
The comparison of antioxidant activities in different co-fermented Cabernet Sauvignon wines.

<table>
<thead>
<tr>
<th>Wine samples</th>
<th>DPPH (TE)(^a)</th>
<th>ABTS (TE)</th>
<th>FRAP (TE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sc</td>
<td>9.01 ± 0.27c</td>
<td>7.62 ± 0.55c</td>
<td>6.52 ± 0.40b</td>
</tr>
<tr>
<td>SWG5</td>
<td>10.32 ± 0.16 ab</td>
<td>12.59 ± 1.14a</td>
<td>7.13 ± 0.38 ab</td>
</tr>
<tr>
<td>SWG6</td>
<td>9.91 ± 0.29b</td>
<td>12.77 ± 1.52a</td>
<td>7.35 ± 0.46 ab</td>
</tr>
<tr>
<td>SWG11</td>
<td>8.96 ± 0.35c</td>
<td>9.83 ± 0.87ab</td>
<td>6.55 ± 0.23 b</td>
</tr>
<tr>
<td>SWG26</td>
<td>9.16 ± 0.53bc</td>
<td>12.65 ± 1.60a</td>
<td>6.73 ± 0.17b</td>
</tr>
<tr>
<td>SWG27</td>
<td>10.46 ± 0.25a</td>
<td>13.74 ± 2.56a</td>
<td>7.98 ± 0.47a</td>
</tr>
</tbody>
</table>

\(^a\) DPPH, ABTS and FRAP expressed as mM Trolox equivalents (TE).

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wines, as determined by UHPLC-Q-TOF-MS analysis. In addition, co-fermented wines had lower levels of malic acid, an organic acid produced during the fermentation process when sugars enter the TCA cycle through glycolysis. This degradation of malic acid is attributed to the ability of *W. anomalus* strains to more efficiently utilize this compound and utilize it as a sole source of carbon (Corte-Real & Leao, 1990; Saayman & Viljoen-Bloom, 2006). Our studies showed a similar trend to previous studies by Satora (Satora et al., 2014) and Sun (Sun et al., 2022) on apple and kiwi wines, respectively. Furthermore, metabolites such as mannitol, erythritol and tartaric acid were also observed at lower levels in SWG27 wine. This decrease was consistent with reports of some *W. anomalus* strains that can assimilate these polyols, but *S. cerevisiae* and others *W. anomalus* strains cannot (Kim et al., 2013; Zaky et al., 2016). Previous research has stated that non-*Saccharomyces* can degrade more tartaric acid than *S. cerevisiae* in the sequential inoculation (Dutraive et al., 2019). Consistent with these reports, WG27 strain could utilize these specific polyols and use more tartaric acid for its metabolisms. Meanwhile, the SWG27 wine had lower levels of quinic acid and shikimic acid, intermediate metabolites of the shikimate pathway that supplies precursors to phenolic compounds such as phenolic acids and flavonoids (Herrmann & Weaver, 1999; Minebois et al., 2020a). These decrease indicated an intensification of this metabolic branch by WG27 strain. Our hypothesized that metabolites underwent conversion towards phenylalanine metabolism, and subsequently towards phenylpropanoid biosynthesis, was supported by the up-regulation of products such as phenethyl alcohol, flavonoid derivatives, and cinnamic acid derivatives. Zaky (Zaky et al., 2016) also reported that a strain of *W. anomalus* can utilize quinic acid, which was consistent with our findings on WG27 strain. The lower levels of
shikimic acid observed in co-fermentation suggested that *W. anomalus*, except for WG5 strain, could utilize shikimic acid to produce secondary metabolites.

Additionally, pyruvate, a crucial intermediate metabolite in carbon metabolism, can be directed toward catabolic reactions in the TCA cycle or anabolic reactions for the formation of oxaloacetic acid or amino acid biosynthesis (Richter et al., 2015). Pyruvate is an effective precursor feeding agent that can stimulate the biosynthesis of terpene lactones (Kang et al., 2006). The decrease in pyruvate content and higher levels of terpenoids and steroids in SWG27 wine suggested that WG27 strain might promote pyruvate conversion through terpenoid backbone and steroid biosynthesis and metabolism. This wine also has higher levels of mesaconic acid and methyglyoxal, which could serve as precursor intermediate metabolites for synthesizing pyruvate and further enhancing terpenoid backbone and steroid biosynthesis and metabolism.

4.1.3. Vitamins

Vitamins are primordial for certain yeast physiological functions, and intracellular vitamin deficiencies can repress many metabolic pathways and impair growth and fermentation (Evers et al., 2023; Evers et al., 2021; J. Li, Ye et al., 2020). Vitamins act as a versatile coenzyme or substrate for many enzymes involved in reduction-oxidation systems, antioxidant activities, membrane integrity, cellular signaling, cellular protection, and yeast respiration (Evers et al., 2021). Our study observed down-regulated vitamin B₆, also called pyridoxine, in SWG5/Sc and SWG27/Sc did not depend on the chain length, but rather reflected the activation of fatty acid metabolic pathways and effective response to stress and competitive inhibition in the fermentation environment. Especially, the level of caprylic acid was significantly up-regulated in SWG26/Sc and SWG27/Sc, suggesting that WG26 and WG27 strains had better environmental adaptability to resist stress and inhibition. Although caprylic acid has toxic effects on yeast (Legras et al., 2010), it can provoke an adaptive response in yeast cells to resist larger amounts of inhibitor at sub-lethal concentrations (Cabrál et al., 2001).

4.1.4. Nucleotides

Nitrogen availability is crucial for winemaking since organic and inorganic nitrogen affects yeasts growth, fermentation kinetics, and flavor generation, and ultimately the quality and composition of wine (Álvarez-Fernández et al., 2020). Inadequate nitrogen concentrations can result in low concentrations of yeast cell biomass and slow and/or stuck fermentations (Crépin et al., 2012; Álvarez-Fernández et al., 2019). These phenomena can be avoided by the extensive utilization of nitrogen sources by yeast. Purine and pyrimidine bases, ammonium, urea, amino acids, and small peptides can all be used as nitrogen sources during fermentation (Álvarez-Fernández et al., 2019). Therefore, it is hypothesized that the lower level of hypoxanthine in all co-fermentation wines than Sc wine could be related to the stress conditions of nitrogen consumption, thus promoting purine metabolism and providing nitrogen sources to be consumed. In addition, activation of xanthine oxidase to produce uric acid is an effective defense strategy for microorganisms against oxidative stress (Arapitas & Scholz, 2012). Hence, the up-regulation of uric acid in SWG27/Sc suggested that the WG27 strain has higher oxidative stress response than other *W. anomalus* strains.

4.1.5. Amino acids

Amino acids make up 51–92% of the nitrogen assimilated by yeast in grape juice during wine fermentation, but these nitrogen sources do not all equally support growth and fermentation, and the consumption order could be classified into three groups (Crépin et al., 2012; Alvarez-Fernández et al., 2019). The first group (named prematurely consumed) was L-lysine, which was exhausted during the initial hours of fermentation (Crépin et al., 2012). The decrease of L-lysine or its derivative (lysopine) contents in SWG5, SWG26, and SWG27 wines compared to Sc wine indicated that these three strains consumed more nitrogen sources for growth and metabolism than *S. cerevisiae* during the start-up stage of co-fermentation before stress conditions and competitive inhibition. This result suggested that these three strains can play a more important role in co-fermentation and potentially improve wine quality.

The second group (named early consumed) includes L-aspartate, L-glutamate, L-leucine, L-histidine, L-methionine, L-phenylalanine, etc (Crépin et al., 2012). During fermentation, W5G and WG26 strains consumed L-phenylalanine, an aromatic amino acids, faster than *S. cerevisiae*. Crépin et al., (Crépin et al., 2012) also reported that L-phenylalanine consumption was high during the growth phase of the initial fermentation, then decreased and remained at a low level. Yeast metabolism of aromatic amino acids can convert nitrogen compounds into a range of volatile and nonvolatile metabolites, especially an important class of aroma compounds—esters (Richter et al., 2015). Our earlier report indicated that the contents of ethyl esters in SWG5 and SWG26 wines were significantly increased than Sc wine (Wang et al., 2023). Meanwhile, L-homoserine, as a downstream metabolite of L-aspartate, was more assimilated by WG26 and WG27 strains than *S. cerevisiae* during co-fermentation. As L-glutamate downstream metabolites, the
higher assimilation of L- pyroglutamic acid and L-threonine in SWG26/Sc, and L-ornithine in SWG27/Sc, were observed. These results indicated that WG26 and WG27 strains assimilate more available nitrogen sources, which will be conducive to the growth and metabolism of these two _W. anomalus_ strains, and further produce more beneficial metabolites to improve the quality of wine.

As the late consumed group, the levels of other two aromatic amino acids, L-tyrosine and L-tryptophan, were not significantly altered between each type of co-fermentation and _S. cerevisiae_ fermentation alone. This result suggested that _W. anomalus_ strains also consumed L-tyrosine and L-tryptophan but at a lower rate, similar to _S. cerevisiae_ (Álvarez-Fernández et al., 2020). Remarkable was the increase of early consumed amino acids as L-leucine, L-histidine, L-methionine, L-phenylalanine in SWG6/Sc, indicating that WG6 strains have a limited ability to assimilate nitrogen sources than _S. cerevisiae_ in co-fermentation. This limited assimilation ability hinders the related metabolic pathways of WG6 strains, such as transamination reaction, decarboxylation reaction, dehydrogenation reaction and oxidation reaction. In particular, transamination reaction is the initial step in the fermentation process to produce alcohol, aldehyde, acid, ester and other aroma compounds (Wattanakul et al., 2020). Combined with our early finding that the level of ethyl esters decreased in SWG6 wine than Sc (Wang et al., 2023), we deduced that the application of WG6 strain in wine fermentation was limited. Finally, we also found that there was no significantly difference in amino acids contents of SWG11/Sc, suggesting that WG11 strain had a comparable ability to assimilate amino acids as _S. cerevisiae_.

### 4.2. Effect of co-fermentation on secondary metabolism

#### 4.2.1. Aroma compounds

In wine, the main secondary metabolites are aroma compounds and polyphenols that give the wine its aroma and taste, while the contents of other secondary metabolites are relatively low, such as alkaloids and lignans. Consistent with earlier GC-MS results (Wang et al., 2023), we observed a higher accumulation of phenethyl alcohol in SWG27/Sc. Since L-leucine can form isoamyl alcohol and further synthesize isoamyl acetate with acetyl-CoA (Lambrechts & Pretorius, 2000), the non-significant decrease in L-leucine content could explain why the content of isoamyl acetate in co-fermenting wines reported earlier did not increase (Wang et al., 2023). We also observed that all co-fermentation enhanced the level of methylantranilate, which has the typical “foxy” smell in wild American species, but this characteristic flavor is not solely due to its presence (Nelson et al., 1977). Despite this, it is worth noting that methylantranilate also imparts a sweet-fruity, grape-like taste with a distinctly floral perfumery character in Pinot noir wines (Moio & Ettienne, 1995), suggesting that the increase of methylantranilate content may have important sensory impact in _Vitis vinifera_ grapes, such as Cabernet Sauvignon in the present study. Moreover, through tryptophan metabolism, WG27 strain could bio-transform more odorless metabolites into flavor active compounds, such as indole and indole-3-acetaldehyde, thus directly contributing to wine aroma (Álvarez-Fernández et al., 2019). Of course, there needs to be a balance between all the aroma substances in order to enhance the flavor of the wine and thus increase consumer acceptance. It is encouraging to note that our early sensory evaluations have shown that SWG27 wine had the best sensory score of all the co-fermented wines (Wang et al., 2023).

#### 4.2.2. Phenolic compounds in wine fermented with WG27 strain and _S. cerevisiae_

Phenolic compounds have one or more hydroxyl groups attached to an aromatic ring(s), and exhibit multifaceted biological activities, including anti-oxidation, anti-inflammatory, anti-tumor, antiviral and anti-microbial activity (Ma et al., 2020; Sun et al., 2021; Velderrain-Rodríguez et al., 2014). According to a significant study, consumption of red wine above or double above the guidelines might lower your risk of contracting COVID-19 (Dai et al., 2022). Ma (Ma et al., 2020) summarized 23 promising antiviral drug candidates by microbial engineering, especially aromatics, including flavonoids and phenylpropanoid derivatives, as the most abundant and largest antiviral natural products. Anti-oxidation experiments have shown that WG5, WG6 and WG27 strains can significantly increase the antioxidant activity of co-fermented wines, especially WG27 strain. The SWG27 wine had the highest quantity of up-regulated secondary metabolites, with the majority belonging to the phenolic family, as previously mentioned (phenolic acids, phenylpropanoids, flavonoids, anthocyanin, proanthocyanidin, stilbenes, coumarins and more). As reported in the literature (Ma et al., 2020), higher levels of p-coumaric acid, caffeic acid, silan- drin, taxifolin 3-O-acetate (condensation between taxifolin and acetic acid), polydatin (glycosylated form of resveratrol), quercetegatin (quercetin derivatives) in SWG27 wine might have positive antiviral effects. Additionally, we assumed that up-regulated other non-antioxycholic phenolic compounds common in red wine, such as myricitrin, isorhamnetin, syringetin-3-O-beta-D-glucoside, kaempferol 3-O-beta-glucoside (trifolin), chlorogenic acid and salicylic acid, exhibited certain biological activity. We also observed that some non-antioxycholic phenolic compounds, such as tricin (Z. Li, Long et al., 2022), passectanol 3-O-glucoside (Z. Li, Long et al., 2022), umbelliferone (Winstel et al., 2020), robinetin (Morata et al., 2019, pp. 163–176), amelopsin (Flamini & De Rosso, 2019) and hesperidin (Dutra et al., 2018), were substantially up-regulated in SWG27 wine; although these components have been seldom reported in grapes and wine. Given their putative positive effect on human health, it would be worthwhile to explore their presence and behavior throughout co-fermentation. Nevertheless, further studies are necessary to confirm this hypothesis and the beneficial effects of WG27 strain and _S. cerevisiae_ co-fermented wine may extend beyond flavor.

Monomeric and polymeric flavanols (tannins) are another class of phenolic compounds, such as catechin gallate, procyanidin B1,3’-O-gallate, procyanidin B5-3’-O-gallate, proanthocyanidin A2, which were higher levels in SWG27 wine than Sc wine. These bioactive compounds not only stabilize anthocyanins, leading to better color retention, but they also react with each other to form new tannins, influencing the taste and structure of red wines (Arapitsas & Scholz et al., 2012; Herderich & Smith, 2005; Nel, 2018). In this experiment, although no anthocyanins were observed in the differential metabolites, some pigments were up-regulated. Indeed, an increased presence of color intensity in SWG27 wine has been reported in our previous study (Wang et al., 2023). Initially, we noticed that WG27 strain increased the content of vitisin A, which could enhance color stability of wine. This is due to the fact that vitisin A is more stable than anthocyanins and increase anthocyanin reactivity to form new pigments (Arapitsas & Scholz et al., 2012). Furthermore, the up-regulation of quercetagentin not only appeared to be important yellow pigments, but also an effective copigment to stabilize grape anthocyanins (L. Li, Ye et al., 2020; Xu et al., 2015).

#### 4.2.3. Glucosinolates in wine fermented with WG27 strain and _S. cerevisiae_

The methionine metabolism pathway resulted in elevated levels of glucosinolates in SWG27 wine, specifically glucosolvin, glucoiberin, glucorucin and glucoraphanin. At present, it remains unclear whether the glucosinolates levels are sufficient to affect wine properties, since these glucosinolates are only semi-volatile and lack of obvious association with bitterness (Bell et al., 2018). As such, it is unlikely to have a significant impact on flavor. Nonetheless, the health benefits of these glucosinolates have been well-documented (Bell et al., 2018; Flores et al., 2021; Ishida et al., 2014).

### 5. Conclusion

In summary, this paper elaborated on the commonalities and differences among the five _W. anomalus_ in co-fermented wines through kinetic parameters, metabolomics and antioxidant activities. All
W. anomalus strains promoted the process of TCA cycle and purine metabolism by decreasing malic acid and hypoxanthine levels. However, the five W. anomalus exhibited significantly different or even opposite metabolic characteristics in co-fermented wines, particularly in terms of amino acid metabolism and downstream metabolites of shikimate pathway. The WG5, WG26, and WG27 strains showed positive responses to amino acid biotransformation. It is worth noting that WG27 strain brought a large number of differential metabolites to the wine samples, especially secondary metabolites that were not considered in previous studies. These differential metabolites likely affect not only the flavor but also the antioxidant activities of SWG27 wine, as evidenced by the up-regulation of many bioactive polyphenols. Our study has practical implications for the industry. Enologist can take advantage of these specific metabolic properties and develop better fermentation strategies using W. anomalus strains.

CRediT authorship contribution statement

Jun Wang: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. Juanjuan Yan: Conceptualization, Methodology, Formal analysis, Investigation, Data curation. Hengfang Gao: Methodology, Investigation, Data curation. Xia Li: Investigation, Data curation. Zhigang Dong: Resources. Sha Yan: Formal analysis, Visualization. Fei Shi: Conceptualization, Methodology, Supervision.

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

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115229.

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