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Comparative metabolome and transcriptome analyses of the properties of *Kluyveromyces marxianus* and *Saccharomyces* yeasts in apple cider fermentation

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ABSTRACT

This study explored the application of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* (commercial and wild type) in the alcoholic fermentation of *Fuji* apple juice under static conditions. Metabolome analyses revealed that ethyl esters, including ethyl hexanoate, ethyl decanoate, ethyl octanoate, octanoic acid and decanoic acid, were the dominant components in ciders fermented by the *Saccharomyces* yeasts. In the *K. marxianus* ciders, ethyl acetate, hexyl acetate, propyl acetate and acetic acid were the most abundant volatiles, suggesting that the cider fermented by *K. marxianus* might have a fruitier smell. Transcriptome analyses were adapted to gain insight into the differential metabolite patterns between *K. marxianus* and *S. cerevisiae* during cider fermentation. GO and KEGG enrichments revealed that the metabolic pathways of glucose, organic acids and amino acids during cider fermentation were quite different between these two yeasts. The *K. marxianus* strain exhibited a higher rate of glycolysis and ethanol fermentation than did *Saccharomyces* yeasts under oxygen-limited conditions. It also reduced the metabolic flux of acetate into acetyl-CoA and then into the TCA cycle, increasing the syntheses of ethyl acetate and relevant esters, which may affect its cell growth under anaerobic conditions but enriched the taste and variety of aromas in apple cider.

1. Introduction

In traditional cider brewing, spontaneous fermentation performed by indigenous microbiota usually lasts for several weeks when Saccharomyces spp. are the main yeasts responsible for alcoholic fermentation during cider production (Cousin, Le Guellec, Schlusselhuber, Dalmasso, Laplace, & Cretenet, 2017; Valles, Bedrinana, Tascon, Simon, & Madrera, 2007). To accelerate the fermentation process, yeast starters are often added to the must. On an industrial scale, however, ciders are commonly fermented with selected starters, such as Saccharomyces cerevisiae and Saccharomyces bayanus, to ensure reliability and minimize sensory deviations, which concurrently limits the diversity of wine styles and the complexity of their flavours (Wei, Zhang, Yuan, Dai, & Yue, 2019). In recent years, non-Saccharomyces yeasts have received greater attention due to their flavour improvement potential in wines and ciders. In particular, Hanseniaspora uvarum, Metschnikowia pulcherrima, Pichia kudriavzevii and other yeast species are selected to produce alcoholic beverages through pure, simultaneous or sequential co-fermentation with Saccharomyces yeasts (Hranilovic, Gambetta, Jeffery, Grbin, & Jiranek, 2020; Shi, Wang, Chen, & Zhang, 2019; Wei, Zhang, Wang, Ju, Niu, Song, et al., 2020; Yan, Zhang, Joseph, & Waterhouse, 2020).

Kluyveromyces marxianus is a new alternative yeast for wine and cider making that can ferment several carbohydrate substrates into ethanol and grow rapidly (Fonseca, Heinzle, Wittmann, & Gombert, 2008; Sukhang, Choojit, Reungpeerakul, & Sangwichien, 2019; Yamahata, Toyotake, Kunieda, & Wakayama, 2020). As an ethanologenic yeast, K. marxianus is one of the few species that can secrete endogenous polygalacturonase, an important pectolytic glucanase primarily implicated in the solubility of pectin (Serrat, Rodriguez, Camacho, Vallejo,

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Ageitos, & Villa, 2011). Recent work showed that the addition of K. marxianus increased the yield of free-run wine and clearly altered the aroma profile of wine (Rollero, Zietsman, Buffetto, Schuckel, Ortiz-Julien, & Divol, 2018). Furthermore, K. marxianus holds the potential to synthesize additional aroma compounds, such as ethyl acetate and 2-phenylethanol (Morrissey, Etschmann, Schrader, & de Billerbeck, 2015). Recently, Gschaedler et al. investigated the cider production performance of an autochthonous K. marxianus strain isolated from a spontaneous cider fermentation process and found that it produced four times more ethyl acetate than did S. cerevisiae but required a long adaptation phase (Gschaedler, Iniguez-Munoz, Flores-Flores, Kirchmayr, & Arellano-Plaza, 2021). Nevertheless, the metabolic characteristics of K. marxianus in apple cider fermentation are comparatively limited, and its contribution to the composition of apple cider aroma and nonvolatile compounds needs to be investigated.

The organoleptic characteristics of fermented beverages are the main factors influencing consumer acceptance and depend greatly on the aroma compounds and nonvolatile secondary metabolites produced during fermentation (Symoneaux, Guichard, Le Quéré, Baron, & Chollet, 2015; Vidal, Courcoux, Francis, Kwiatkowski, Gawel, Williams, et al., 2004). Untargeted metabolomics based on high-resolution mass spectrometry is an effective tool for fingerprinting aroma compounds and secondary metabolites in apple ciders. The aim of the present study was to compare the volatile and nonvolatile components in ciders fermented by selected *K. marxianus* and traditional *Saccharomyces* yeasts. Transcriptome analyses were performed to track the molecular mechanisms underlying the metabolic differences.

2. Materials and methods

2.1. Yeast strains and apple juices

One non-*Saccharomyces* yeast and two *S. cerevisiae* strains were used in this study. The *K. marxianus* (KM) strain Fim-1 was obtained from Shanghai Engineering Research Center of Industrial Microorganisms (Shanghai, China). Commercial *S. cerevisiae* strain SY (AQ) was purchased from Angel Yeast Co., Ltd. (Yichang, China). The widely used laboratory strain *S. cerevisiae* S288C (SC) was sourced from ATCC (204508). Apple juices (CK) obtained by crushing *Fuji* apples were filtered with diatomite (Celite, 535RV, Linjiang, China), and then the soluble solid content (SSC) was adjusted to 15.0° Brix with sucrose. After that, apple juices were pasteurized for 30 min at 68 °C.

2.2. Cider fermentations

Yeast starters were incubated in liquid YPD overnight at 30 °C with a rotation speed of 220 rpm. Yeast cells were collected by centrifugation and suspended in sterile water. For each yeast, cider fermentations were conducted in triplicate with 500 mL-flasks containing 400 mL apple juice supplemented with 50 mg/L potassium metabisulfite. The fermentations were initiated by adding yeast starters at a final optical density (OD_{600nm}) of 0.2 and maintained at 30 °C under static conditions until the total soluble solids decreased to approximately 8.0 °Brix. The fermentation periods were 66, 72 and 216 h for the AQ, SC and KM strains, respectively. The fermented samples were centrifuged at 10,000 rpm for 10 min to separate the supernatants and yeast cells for further biochemical, transcriptional and metabolomic analyses. Total titratable acids in the ciders were determined by titration with NaOH and represented as g/L malic acid. The colour index was analysed by a UV–Vis spectrophotometer at a wavelength of 420 nm. The contents of ethanol, glucose, sucrose and fructose were quantified on an Agilent 1260 Infinity HPLC (Agilent Technologies, Santa Clara, CA, USA) fitted with a refractive index detector using a MetaCarb 87H column (300 mm × 4.6 mm). The column temperature was set at 35 °C, and 5 mM H_2SO_4 (pH 2.0) was used as the mobile phase at a flow rate of 0.6 mL/min.

2.3. GC–MS analysis of volatile components

Volatile compounds in the apple juice and ciders were extracted according to the headspace solid phase microextraction (HS-SPME) method described previously (Villière, Arvisenet, Lethuaut, Prost, & Sérot, 2012). Briefly, 1.5-mL samples were added to the headspace bottle and equilibrated for 15 min at 50 °C. Subsequently, a 50/30 μm DVB/CAR on PDMS fibre was plugged into the headspace bottle for 30 min at 50 °C to trap the volatiles.

To analyse the volatile compounds, HS-SPME extracts were desorbed at 260 °C in the injection port of an Agilent 7890B GC (Agilent Technologies, USA) equipped with a DB-wax column (30 × 0.25 mm, 0.25 μm), a flame ionization detector, and a quadripolar mass spectrometer (Agilent Technologies, 5977B, USA). Helium carrier gas flowed at a constant rate of 1.0 mL/min. The column temperature was held at 40 °C for 5 min, rose to 220 °C at a rate of 5 °C/min, and then increased to 250 °C in 2.5 min. Compounds were identified by comparing their mass spectra with the standard spectra in the reference database (NIST 14). All the volatile compounds were semiquantified by the relative chromatographic peak areas using 2-octanol as the internal standard.

2.4. UPLC/MS-based metabolomics analysis

Nonvolatile compounds in the apple juice and ciders were determined by UPLC/ESI-MSn in both ESI positive and ESI negative ion modes (Hranilovic, Gambetta, Jeffery, Grbin, & Jiranek, 2020). The UPLC separations were executed on a Thermo Vanquish system (Thermo Scientific, San Jose, CA, USA) equipped with an ACQUITY UPLC HSS T3 column (150 mm × 2.1 mm, 1.8 μm) (Waters, Milford, MA, USA) maintained at 40 °C. The mobile phases were 0.1% formic acid–water (A1) and 0.1% formic acid-acetonitrile (B1) for the positive ion mode and 5 mM aqueous ammonium formate (A2) and acetonitrile (B2) at a flow rate of 0.25 mL/min for the negative ion mode. The linear gradients for solvent B (v/v) were as follows: 1 min, 2% B1/B2; 9 min, 50% B1/B2; 12 min, 98% B1/B2 (maintained for 1.5 min); 13.5 ~ 14 min, 2% B1/B2 (maintained for 6.0 min). The ESI-MSn determinations were conducted on a Thermo Q Exactive HF-X mass spectrometer (Thermo Scientific, USA) with spray voltages of 3.5 kV (positive mode) and −2.5 kV (negative mode), and the capillary temperature was maintained at 325 ℃. The mass spectra were scanned at a mass resolution of 70,000 over a mass range of m/z 81 to 1000. The data-dependent MS/MS acquisition was performed with higher energy collisional dissociation (HCD) scanned at a normalized collision energy of 30 eV.

2.5. RNA extraction, cDNA library construction and RNA sequencing

For transcriptome sequencing, yeast cells were collected after incubation for 12 h and at the end of fermentation (8.0 °Brix). Total RNA was isolated using TRIzol Reagent (Thermo Scientific, USA) following the manufacturer's protocol and quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA). Then, sequencing libraries were generated using the VAHTS mRNA-seq V3 Library Prep Kit for Illumina (Vazyme Biotech, Nanjing, Jiangshu, China). Transcriptome sequencing was performed on a NovaSeq 6000 (Illumina, San Diego, CA, USA) by Personal Biotechnology Co., Ltd. (Shanghai, China). All three independent replicates were analysed.

2.6. Bioinformatics analysis

Filtered clean reads for transcriptome sequencing were mapped to the reference K. marxianus (https://www.ncbi.nlm.nih.gov/assembly/GCA_001854445.2) and S. cerevisiae (https://www.ncbi.nlm.nih.gov/assembly/GCA_003086655.1) genomes using HISAT2. The gene expression levels were normalized with the FPKM (fragments per kilobase per million fragments) method. Differentially expressed genes (DEGs) were identified using the DESeq R package, and a P value < 0.05

with a twofold change was considered to be significant. DEGs were mapped to the Gene Ontology (GO) and KEGG databases to identify their functions and biological processes. The *P* values were calculated from the hypergeometric distribution.

2.7. Statistical analysis

All results were expressed as the mean (±standard deviation) of three replications. Data analyses were performed using SPSS Statistics version 19 (SPSS Inc., Chicago, IL, USA). To compare the differences in chemical compositions, a one-way analysis of variance (ANOVA) was conducted with the Tukey test at a significance level of $p < 0.05$. Principal component analysis was performed using Origin 2017 (OriginLab, Northampton, MA, USA).

3. Results

3.1. Volatile compounds in the ciders

A total of 102 kinds of volatile compounds were detected from the apple juice and the three ciders. Specifically, 48 volatile compounds were detected in apple juice, while in the ciders fermented with KM, SC and AQ, there were 48, 58 and 60, respectively, types of volatiles found. These flavour compounds were mainly classified into alcohols, acids, esters, and carbonyls (Table 1). Principal component analysis (PCA) was applied to reduce the dimensionality of volatile components in different samples (Fig. 1). As shown in Fig. 1, PC1 and PC2 accounted for 52.39% and 26.02% of the total variance, respectively, and principal components in the three ciders were clearly distinguished. However, unlike the SC and AQ ciders, the principal components in the KM cider were well correlated with those of the apple juice. Of the detected volatile compounds, 1-hexanol, ethyl hexanoate, hexyl acetate, 2-phenylethanol and ethyl decanoate made large contributions to the variance of PC1.

Overall, fermentation of apple juice with yeast markedly increased the contents of volatile components and changed the aroma composition. The major components of apple juice were aldehydes and alcohols, which accounted for 43.6% and 29.8% of the total volatiles, respectively. In cider samples, alcohols (63.3–77.8%) and esters (17.3–30.4%) were the dominant volatiles. In addition, yeast fermentations significantly increased the contents of higher alcohols, esters and volatile acids (Table 1), indicating that the dominant aroma of the apple juices was transformed from green and fruity to bouquet, fruity and fatty. Among the three ciders, there were likewise significant differences in the compositions of esters and volatile acids. Ethyl esters (ethyl hexanoate, ethyl decanoate, and ethyl octanoate), octanoic acid and decanoic acid were the principal components in ciders fermented by the *Saccharomyces* yeasts SC and AQ. In the KM ciders, acetate esters (ethyl

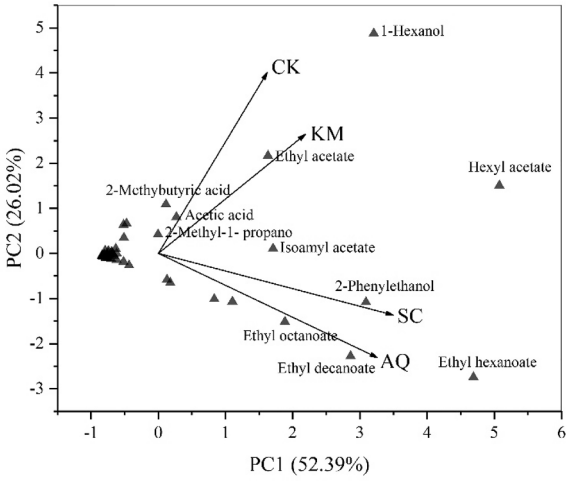

Fig. 1. PCA biplot of the volatile components in the apple juice and cider samples. CK, apple juice; KM, the *K. marxianus* Fim-1 fermented cider; SC, the *S. cerevisiae* S288C fermented cider; AQ, the *S. cerevisiae* SY fermented cider.

Table 1
Volatile compounds in different ciders.

Compounds	Concentration (μg/L)				Odour threshold (mg/L)	Description
	CK	KM	SC	AQ		
Alcohols						
1-Hexanol	171.8 ± 2.1	225.0 ± 4.2	257.5 ± 3.5	204.7 ± 8.2	8	Green, herb
2-Methyl-1-propanol	1.6 ± 0.1	201.4 ± 12.8	61.5 ± 14.8	40.0 ± 7.8	40	Fusel, alcohol
2-Phenylethanol	nd	190.4 ± 10.0	656.4 ± 66.2	354.7 ± 7.4	14	Green
1-Propanol	nd	23.9 ± 2.4	19.6 ± 5.6	23.7 ± 2.1	206	Alcohol, pungent
1-Butanol	23.7 ± 4.0	20.7 ± 0.0	nd	23.3 ± 1.1	150	Medicine, fruit
Fatty acids						
Acetic acid	7.9 ± 2.5	279.8 ± 30.2	48.3 ± 7.3	73.6 ± 19.2		Sour
2-Methyl butyric acid	38.7 ± 1.8	66.7 ± 3.8	48.9 ± 3.1	69.7 ± 3.3	0.033	Cheese
Octanoic acid	nd	Nd	268.8 ± 24.8	410.7 ± 48.6	0.5	Rancid, harsh, cheese, fatty acid
Decanoic acid	nd	Nd	170.2 ± 26.2	485.5 ± 60.7	1	Fatty, unpleasant
Esters						
Ethyl hexanoate	9.5 ± 0.9	Nd	650.9 ± 50.6	1351.9 ± 54.2	0.005	Fruity, green apple, floral, violet
Hexyl acetate	44.8 ± 2.3	756.5 ± 28.1	445.4 ± 12.5	858.1 ± 15.1	1.5	Pleasant fruity, pear
Ethyl decanoate	nd	Nd	289.7 ± 30.1	1253.3 ± 137.6	0.2	Fruity, fatty, pleasant
Ethyl octanoate	nd	19.7 ± 1.9	280.1 ± 13.5	758.0 ± 99.3	0.002	Fruity, pineapple, pear, floral
Isoamyl acetate	nd	370.9 ± 13.2	196.5 ± 17.7	421.7 ± 52.7	0.03	Banana, fruity, sweet
Ethyl 9-decenoate	nd	Nd	117.8 ± 23.5	240.9 ± 15.2	0.1	Green, fruity, fatty
Ethyl acetate	2.8 ± 0.2	821.2 ± 67.8	68.6 ± 5.5	93.9 ± 8.5	7.5	Fruity, sweet
Ethyl butyrate	14.6 ± 0.2	13.5 ± 3.1	21.6 ± 3.8	19.4 ± 2.6	0.02	strawberry, fruity
Ethyl 2-methylbutanoate	24.0 ± 0.9	3.6 ± 0.1	6.4 ± 0.8	5.7 ± 0.3		Apple
Propyl acetate	5.0 ± 0.4	720.7 ± 95.9	nd	nd	4.7	Fruity, sweet
Aldehydes						
(E)-2-Hexenal	210.3 ± 0.6	Nd	nd	nd		
Hexanal	157.1 ± 3.8	Nd	nd	nd		Grass, fatty
Benzaldehyde	94.1 ± 1.9	Nd	8.2 ± 0.6	nd	2	Bitter almond

CK, apple juice; KM, SC and AQ, the ciders fermented with *K. marxianus* Fim-1, *S. cerevisiae* S288C and *S. cerevisiae* SY, respectively;
nd, not detected;
The odour threshold and description of volatile compounds referred to (Picinelli Lobo, Pando Bedrinana, Rodriguez Madrera, & Suarez Valles, 2021; Qin, Petersen, & Bredie, 2018; Shi, Wang, Chen, & Zhang, 2019).

acetate, hexyl acetate, propyl acetate) and acetic acid were the most abundant volatiles. These results suggest that the cider fermented by *K. marxianus* might have a sweeter smell than that fermented by *Saccharomyces* yeasts. Compared to the laboratory *Saccharomyces* strain S288C, the industrial strain AQ produced markedly more fatty acids and esters during fermentation.

3.2. Nonvolatile compounds in the ciders

Some of the nonvolatile compounds detected in different samples, including flavonoids, phenolic acids, organic acids and amino acids, are listed in Table 2. Consistent with the titration of titratable acid, the contents of phenolic acids and organic acids in all cider samples apparently increased after fermentation. However, KM cider had the highest content of titratable acid (Table 3). Of the identified organic acids, the contents of isocitric acid, succinic acid, phenyl lactate and malic acid increased significantly in all ciders after fermentation. Although the profiles of nonvolatile components in the three ciders were quite similar, the contents of these nonvolatiles were significantly different. Compared with the ciders made with *Saccharomyces* yeasts, the KM cider had relatively higher contents of hesperetin, quercetin, caffeic acid, salicylic acid and amino acids.

In cider fermentation, amino acids in fruit juice provide the main nitrogen sources for yeast growth and are also involved in the synthesis and metabolism of volatile compounds (Callejón, Tesfaye, Torija, Mas, Troncoso, & Morales, 2007). To determine the differential metabolite profiles of amino acids between *K. marxianus* and *S. cerevisiae* during cider fermentation, all relevant metabolites detected in ciders were mapped into the metabolic pathways of organic acids and amino acids. As shown in Fig. 2, except for malate, the metabolites involved in the TCA cycle, amino acid biosynthesis and metabolism pathways were apparently higher in KM cider than in SC and/or AQ ciders. This may be because *K. marxianus* might have a higher metabolic flux into the TCA cycle and amino acid biosynthesis (metabolism) than do *Saccharomyces* yeasts, which reduced the intake and assimilation of amino acids from apple juice. The differential metabolite profile of amino acids in *K. marxianus* may contribute to improving the quality and nutrition of ciders.

Table 2
Nonvolatile compounds determined by UPLC-MS in apple juice and ciders.

Compounds	*m/z*	Peak area CK	KM vs CK	SC vs CK	AQ vs CK
Flavonoids					
Catechin	289.07	1,846,641	0.18	0.70	1.03
Phloretin	275.09	750,658,765	0.78	1.05	1.00
Naringenin	273.08	3,411,617	0.85	1.18	0.77
Isorhamnetin	317.07	1,982,633	1.88	4.87	6.87
(-)-Epigallocatechin	306.08	13,525,711	2.36	5.35	0.63
Kaempferol	287.06	931,413	4.02	3.86	4.48
Hesperetin	302.08	653,032	10.11	3.86	1.97
Quercetin	303.05	2,450,348	34.61	2.41	3.30
Phenolic acids					
Chlorogenic acid	353.09	5,459,505	0.65	6.21	2.62
trans-Ferulic acid	195.07	75,598,911	1.84	2.11	1.61
Gallic acid	171.03	50,302,811	2.19	3.49	6.00
Caffeic acid	181.05	17,749,226	14.14	2.44	1.92
Salicylic acid	137.02	18,463,606	19.51	7.72	8.59
acids					
Glutaric acid	115.04	499,485,594	0.30	0.34	0.51
2-Oxoglutarate	129.13	131,340,847	1.37	1.04	0.89
Citric acid	191.02	17,072,235	1.69	0.30	0.42
γ-Aminobutyric acid	104.07	1,807,997,231	1.80	0.08	0.32
Butyric acid	88.08	416,198,517	4.38	4.65	3.70
L-Malic acid	133.01	15,666,278	5.26	5.92	19.89
Phenyllactate	165.05	47,847,027	8.97	17.35	11.25
Succinic acid	117.02	206,668,652	74.30	31.21	25.22
Isocitric acid	192.03	1,127,666	169.53	463.64	10.00
Amino acids					
L-Proline	116.07	1,445,418,400	10.20	3.03	5.55
L-Tryptophan	203.08	70,271,875	1.13	0.94	0.94
L-Threonine	120.07	1,756,446,063	0.63	0.24	0.42
L-Aspartic acid	132.03	6,055,388,129	0.48	0.01	0.11
L-Glutamic acid	146.04	549,100,546	0.41	0.11	0.44
L-Serine	105.04	1,418,100,386	0.17	0.00	0.00
L-Valine	118.09	3,185,255,281	0.16	0.07	0.07
L-Lysine	147.11	317,019,408	0.07	0.07	0.03

CK, apple juice; KM, SC and AQ, the ciders fermented with *K. marxianus* Fim-1, *S. cerevisiae* S288C and *S. cerevisiae* SY, respectively.

Table 3
Physicochemical parameters of apple juice and apple ciders.

Index	Groups CK	KM	SC	AQ
Titratable acid/ $g \cdot L^{-1}$	2.18 ± 0.02^{d}	3.77 ± 0.04^{a}	2.70 ± 0.03^{c}	2.83 ± 0.02^{b}
Color (420 nm)	0.48 ± 0.00^{a}	0.34 ± 0.01^{b}	0.24 ± 0.00^{d}	0.29 ± 0.00^{c}
Ethanol/$g \cdot L^{-1}$	ND	44.96 ± 0.39^{a}	45.62 ± 1.19^{a}	45.62 ± 0.59^{a}
Sucrose	58.41 ± 0.17	ND	ND	ND
Glucose	28.54 ± 0.90^{a}	4.20 ± 0.43^{d}	6.05 ± 0.34^{c}	7.68 ± 0.62^{b}
Fructose	81.74 ± 1.79^{a}	48.07 ± 1.37^{c}	52.53 ± 0.79^{b}	48.06 ± 1.04^{c}

Means with different lowercase letters in the same row are significantly different (Tukey, $p < 0.05$).
CK, apple juice; KM, SC and AQ, the ciders fermented with *K. marxianus* Fim-1, *S. cerevisiae* S288C and *S. cerevisiae* SY, respectively.

3.3. Gene Ontology (GO) and KEGG pathway analyses of differentially expressed genes

DEGs of the three yeasts were analysed by comparing the gene expression profiles between the initial and late stages of the fermentation process. There were 1487, 631 and 932 genes that were differentially expressed in the KM, SC and AQ strains at the two fermentation stages, respectively. To determine the biological functions of these DEGs, all DEGs were subjected to GO analysis, and the major classifications are shown in Fig. 3A. In *K. marxianus,* the DEGs were mainly enriched in acid (carboxylic acid, organic acids and oxoacids), amino acid and organonitrogen compound metabolic processes, while the DEGs in both *Saccharomyces* yeasts were significantly enriched in carbohydrate metabolic, oxidation–reduction and rRNA-related processes. GO cluster analyses suggested that the higher contents of amino acids and organic acids in KM cider were closely related to its anabolic pathways.

To further specify which biological pathways differed between KM and *Saccharomyces* strains, the DEGs were mapped to the terms in the KEGG database. As shown in Fig. 3B, carbohydrate metabolism, amino acid metabolism and transcription were the major pathways that were significantly different among the three yeast strains. In *K. marxianus*, DEGs were significantly enriched in pyruvate and amino acid (lysine, glycine, aspartate, etc.) metabolic pathways. This might be a contributing factor to the higher contents of amino acids in KM-fermented cider than in *Saccharomyces* yeast ciders (Table 2). As indicated by the GO analyses, the DEGs in the SC and AQ strains were enriched into clusters with similar biological functions, but they were classified into different biological pathways in the KEGG pathway enrichment analyses. In particular, in the SC strain, genes for glycolysis, citrate cycle and fatty acid degradation pathways were significantly differentially expressed at the two fermentation stages. In the case of the AQ strain, however, DEGs were enriched in the RNA polymerase and ribosome biogenesis pathways.

To compare the fermentation patterns of the *K. marxianus* and

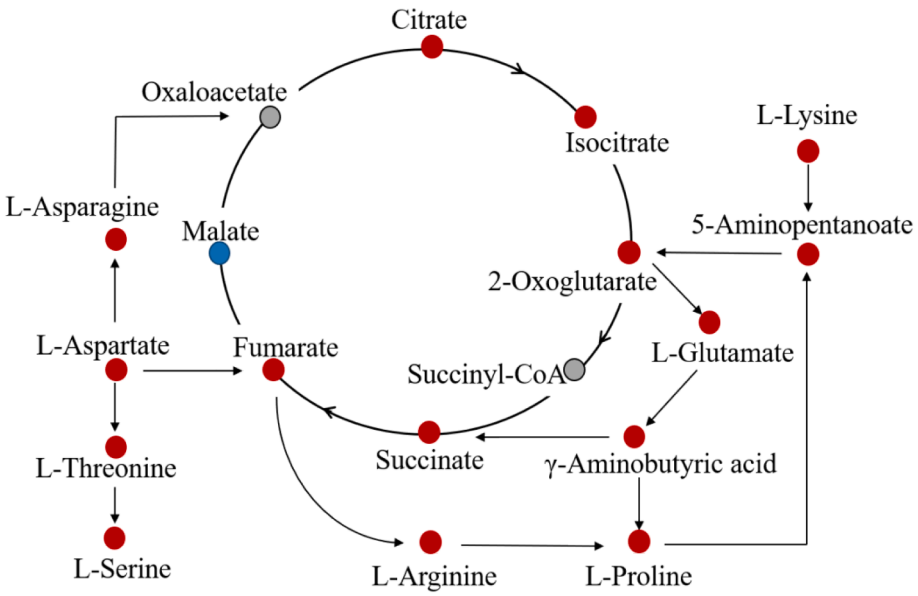

Fig. 2. Analysis of organic acid and amino acid metabolic pathways in different cider samples. Red colour indicates that metabolite content in the KM cider was higher than in the SC cider and/or the AQ cider. Blue colour indicates that the content in the KM cider was lower than in the SC cider and/or the AQ cider. Gray colour: not detected. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Saccharomyces yeasts in cider brewing, the expression levels of the genes involved in central carbon metabolism and ethanol and ester syntheses were further analysed. Fig. 4 shows the fold changes in expression of the key genes involved in glycolysis and pyruvate metabolism pathways for the three yeast strains during cider fermentation. In yeast, hexokinase, 6-phosphofructokinase and pyruvate kinase are the three key rate-limiting enzymes in the glycolytic pathway. The results showed that the expression levels of these key genes coding for hexokinase HXK2 (RAG5), phosphofructokinases PFK1 and PFK2, and pyruvate kinase PYK1 were markedly higher in the KM strain than in *Saccharomyces* yeasts, which suggests that the KM strain might have a higher rate of glycolysis. In addition, alcohol dehydrogenase genes, including *ADH1*, *ADH2* and *ADH6,* were significantly upregulated in the KM strain, while the pyruvate dehydrogenase subunit gene *PDB1*, aldehyde dehydrogenase genes *ALD2* and *ALD6*, and acetyl-CoA synthetase genes *ACS1* and *ACS2* were apparently downregulated, indicating that *K. marxianus* tended to produce ethanol under anaerobic conditions rather than to produce acetyl-CoA. In contrast, in addition to *ADH1*, the genes involved in acetyl-CoA synthesis pathways, including *PDB1*, *ALD2*, *ALD6*, *ACS1* and *ACS2,* were more highly expressed in the *Saccharomyces* yeasts. This result indicates that the central carbon metabolic flux in *S. cerevisiae* was synchronous with ethanol fermentation and acetyl coenzyme synthesis in the initial fermentation stage. In yeast, ethyl acetate is commonly synthesized by esterification of ethanol with acetyl-CoA, which is catalysed by alcohol acetyltransferase (Gethins, Guneser, Demirkol, Rea, Stanton, Ross, et al., 2015). Reducing the metabolic flux of acetyl-CoA into the TCA cycle increases the production of ethyl acetate (Loser, Urit, Forster, Stukert, & Bley, 2012). Therefore, the lower expression of citrate synthase genes CIT1 and CIT3 in the KM strain may contribute to greater accumulation of ethyl acetate in the KM cider (Table 1).

4. Discussion

A typical cider-making fermentation process includes three stages: oxidation, alcoholic fermentation and malolactic fermentation. The oxidative process is the main stage for the production of fruity and floral aromatic compounds, which dominate in cider fermented by non-*Saccharomyces* yeasts (Dierings, Braga, da Silva, Wosiacki, & Nogueira, 2013). Consequently, non-*Saccharomyces* yeasts were good choices for increasing the diversity of cider flavour.

Volatile compounds, organic acids and phenols are the principal components contributing to cider flavour (Symoneaux, Baron, Marnet, Bauduin, & Chollet, 2014). The majority of higher alcohols are byproducts of yeast amino acid and sugar catabolism via the Ehrlich pathway, while esters are produced by the esterification of alcohols with fatty acids (Lorenzini, Simonato, Slaghenaufi, Ugliano, & Zapparoli, 2019). K. marxianus or Saccharomyces yeasts were able to increase the contents of higher alcohols (fusel alcohols), fatty acids and esters during apple juice fermentation. Hexyl acetate, hexanal, 2-methylbutyl acetate and (E)-2-hexenal exhibit high odour activity values (OAVs) that contribute appreciably to the characteristic aroma of apple juice (Valppil, Fan, Zhang, Rouseff, 2009). In this study, the hexyl acetate content increased significantly after fermentation with the KM, SC or AQ strain. Ethyl acetate is the most common ester to play a key role in the perception of fruitiness in young wines (Braga, Zielinski, Silva, de Souza, Pietrowski Gde, Couto, et al., 2013). Among the three ciders, KM cider had the highest content of ethyl acetate, which suggests that the cider fermented by *K. marxianus* may feature a fruitier aroma.

Phenolic compounds are the main flavour compounds affecting the quality and sensory characteristics (colour and mouthfeel) of ciders (Laaksonen, Kuldjarv, Paalme, Virkki, & Yang, 2017). In cider-making, the addition of apple phenolic extracts can improve the colour and perception of the bitterness and astringency of ciders (Benvenutti, Bortolini, Nogueira, Zielinski, & Alberti, 2019). Our results showed that the chromatic value of apple juice decreased significantly after fermentation, and KM cider exhibited the darkest colour among the three ciders (Table 3). As the contents of phenolic acids and organic acids increased after yeast fermentation, the ciders became richer in taste than the unfermented apple juice (Table 2).

K. marxianus is a Crabtree-negative yeast that tends to produce ethanol when oxygen is limited (Nambu-Nishida, Nishida, Hasunuma, & Kondo, 2018). In contrast, S. cerevisiae is a Crabtree-positive model yeast using glycolysis as the terminal electron acceptor in the presence of excessive oxygen (Imura, Nitta, Iwakiri, Matsuda, Shimizu, & Fukusaki, 2020). Our transcriptome data suggested that the rate of glycolysis was higher in *K. marxianus* than in *S. cerevisiae* strains in the initial fermentation stage. However, the metabolic flux in *K. marxianus* was directed to ethanol fermentation rather than the acetyl-CoA synthesis that could support cell growth. In *S. cerevisiae,* both ethanol fermentation and acetyl-CoA synthesis pathways were highly active. This was

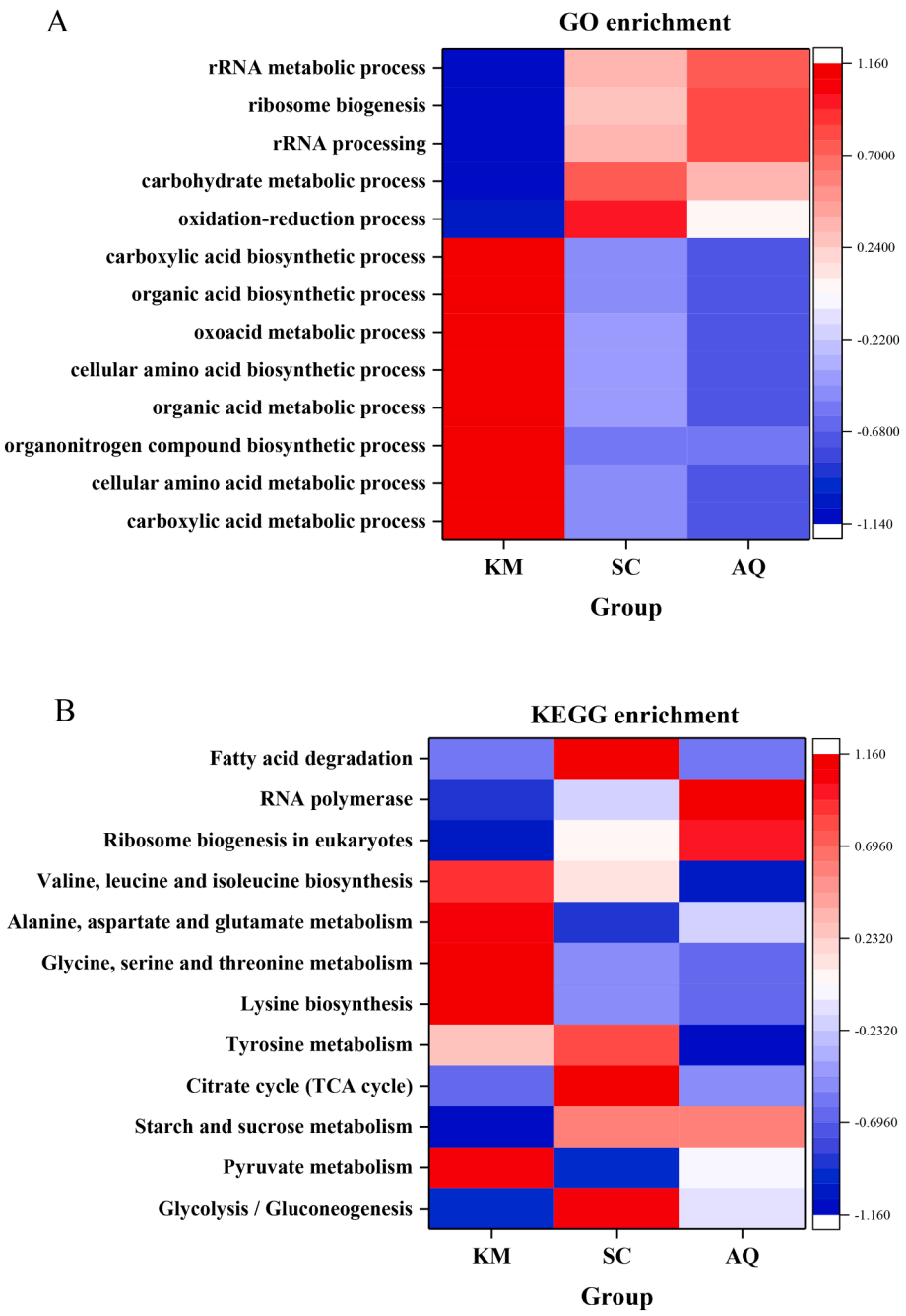

Fig. 3. GO (A) and KEGG (B) enrichment analyses of DEGs in the KM, SC and AQ yeasts during cider fermentation. KM, DEGs of the *K. marxianus* strains; SC, DEGs of *S. cerevisiae* S288C; AQ, DEGs of the *S. cerevisiae* SY strain. The colours (blue to red) represent the p values ($-\log_{10}$)-normalized by the z scores. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

probably why *K. marxianus* required a longer period for cider fermentation than did *S. cerevisiae*. Interestingly, two acetyl-CoA synthetase genes, *ACS1* and *ACS2*, were downregulated in *K. marxianus* during cider fermentation, which contributed to the syntheses of acetate and relevant esters.

5. Conclusions

The non-*Saccharomyces* yeast *K. marxianus*, as well as the two *S. cerevisiae* strains S288C and SY, were used to ferment *Fuji* apple juice under static conditions. Yeast fermentations significantly altered the volatile and nonvolatile profiles of apple juice, with the contents of aldehydes sharply decreasing and the higher alcohols, fatty acids, esters, phenolic acids and organic acids markedly increasing. In apple juice, aldehydes and alcohols were the major aroma components. In contrast, alcohols and esters were the principal components in apple ciders. Ciders fermented with *Saccharomyces* yeasts, especially the AQ strain, contained more ethyl esters, octanoic acid, and decanoic acid. In contrast, *K. marxianus* produced more acetate esters and acetic acid during cider fermentation, which enriched the taste and variety of aromas of apple cider. Transcriptome analyses revealed the differences in activity of metabolic pathways for glucose, organic acids and amino acids during cider fermentation between *K. marxianus* and *Saccharomyces* yeasts. The *K. marxianus* strain exhibited a higher metabolic flux of glycolysis and ethanol fermentation than did *Saccharomyces* yeasts in the initial fermentation stage. In addition, it reduced the metabolic flux

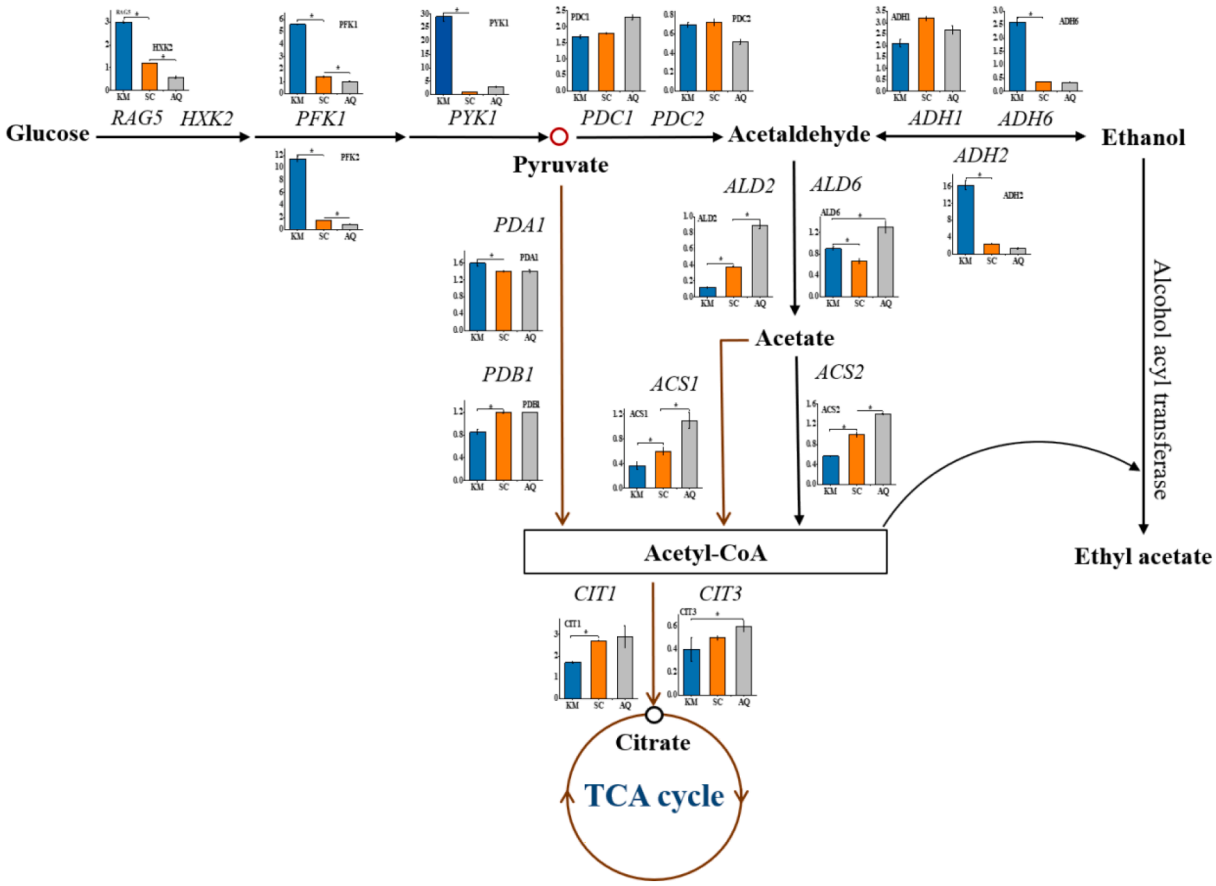

Fig. 4. KEGG pathway analyses of the expression of genes involved in central carbon metabolism and ethanol and ester synthesis in the KM, SC and AQ strains. Red lines, mitochondrial pathways; black lines, cytoplasmic pathways. Column graphs: ordinate column, fold change of gene expression between the initial and late stages of yeast fermentation; x-coordinate, different yeast strains. The relative expression levels in the KM (blue columns), *S. cerevisiae* S288 (orange columns) and SY (grey column) strains are the ratios of the gene expression at the initial stage divided by that in the late stage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of acetyl-CoA synthesis from acetate and acetyl-CoA into the TCA cycle to increase synthesis of ethyl acetate and relevant esters.

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.

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