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ORIGINAL PAPER

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COMPARISON OF FERMENTATION PERFORMANCE AND METABOLITES OF WATER KEFIR GRAINS

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ABSTRACT

Background. Water kefir is a kind of beverage from a brown sugar solution fermented by water kefir grains. It has probiotic functions and unique flavour, and is widely popular in the international market. However, there are few comparative studies on water kefir grains sourced domestically and internationally. Therefore, the purpose of this study was to compare the fermentation performance and metabolites of 6 different water kefir grains from different sources in brown sugar water, in order to provide a theoretical basis for subsequent functional studies and to identify advantages for the development of water kefir.

Methods. The fermentation characteristics of 6 kinds of water kefir grains (HY, GS, LS, SJ, JW, M339) were studied by measuring the wet weight of water kefir grains and the pH value, acidity, polysaccharide yield and antibacterial activity of the fermentation solution. Then the metabolites were analysed by GC-MS. Finally, an orthogonal partial least squares discriminant score (OPLS-DA) was used to identify the differences among the groups.

Results. Among the 6 kinds of water kefir grains, JW had the fastest proliferation, SJ produced more acid and its fermentation broth had the best inhibition effect on *E. coli*, and the yield of exopolysaccharides of LS was the highest. A total of 131 metabolites were detected in 6 kinds of water kefir, including 22 kinds of carbohydrates, 35 kinds of esters, 29 kinds of acids, 10 kinds of alcohols, 8 kinds of nucleosides, 6 kinds of amino acids, 4 kinds of ethers and aldehydes, and 17 kinds of other organic compounds. OPLS-DA showed significant variation among different water kefir groups.

Conclusion. Determination of several important indicators of water kefir grain fermentation and GC-MS analysis of metabolites can clearly identify the differences of water kefir grains from different sources, which is conducive to its targeted development and utilisation.

Keywords: water kefir grain, fermentation performance, metabolites, gas chromatography-mass spectrometry

INTRODUCTION

Water kefir is produced by adding water kefir grains to a mixture of sugar, dried figs or fruit, and vegetable juice, undergoing natural fermentation to produce a slightly acidic, alcoholic beverage rich in beneficial bacteria (David et al., 2014).

Water kefir grains are a kind of elastic, transparent colloidal grain, produced by the interaction of bacteria (Lactic acid bacteria and acetic acid bacteria) and yeast in the extracellular polysaccharide matrix (Waldherr et al., 2010), and their colour is easily affected by the

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colour of fermentation substances (Guzel-Seydim et al., 2021). These grains vary in size, ranging from millimetres to centimetres, and exhibit differences in bacterial composition and proliferation conditions based on their sources (Arslan, 2015).

Active water kefir grains can grow, divide and transmit their characteristics to their offspring, and the increase of their number depends on the division and proliferation of the original water kefir grains (Prado et al., 2015). Carbon source (mostly sucrose) and nitrogen source (new fruit or dried fruit) are the core of microbial growth, metabolism and fermentation in water kefir grains, and the key lies in nutrient interaction and metabolite exchange among microorganisms in the grains (Stadie et al., 2013).

Recent studies have found that there is a mutually beneficial symbiotic relationship between lactic acid bacteria and yeast. The organic acids secreted by lactic acid bacteria can reduce the pH value in the environment and promote the growth of yeast (Ponomarova et al., 2017). Yeast can provide polypeptides and amino acids for microorganisms, such as lactic acid bacteria, and can also secrete β-D-furanosidase to hydrolyze sucrose into glucose and fructose, so that lactic acid bacteria and acetic acid bacteria can use these monosaccharides for their own metabolic activities. hey can also use these monosaccharides as substrates to produce ethanol and CO_2 through glycolysis (Arslan, 2015). Under the influence of *S. cerevisiae*, the expression levels of proteins related to amino acid, carbohydrate, nucleotide metabolism and cell wall synthesis of *L. hordei* were significantly increased (Xu et al., 2019b).

The metabolites of water kefir grains were different due to different fermentation substrates, but in general, they are mainly lactic acid, ethanol, carbon dioxide and a small amount of acetic acid, glycerol, mannitol and various other fermentation products (David et al., 2014), These can make water kefir to possess a unique flavour and aroma, through esters providing a fruit aroma, glycerol affecting texture, etc. (Puerari et al., 2012). *L. hordei* can use pyruvate to produce diacetyl, acetyl, and 2, 3-butanediol, thereby adjusting the sensory characteristics of water kefir (Xu et al., 2019a). Laureys and De Vuyst (2017) also found isoamyl acetate, isoamyl alcohol, ethyl acetate, 2-methyl-1-propanol, ethyl caprylate, ethyl caproate and other volatile esters and higher alcohols in water kefir. Ma et al. (2024) found marker metabolites such as O-acetylserine and β-alanine by using non-targeted metabolomics methods, which are involved in various important metabolic pathways during fermentation.

Currently, there have been limited studies on kefir grains in China, concerned only with microbial composition and development, and the fermentation characteristics and metabolism of water kefir grains are less studied. Therefore, this paper aims to measure key fermentation indicators of kefir grains in various regions of China and abroad, and analyse their metabolites using GC-MS. The objective is to identify and compare any differences between them, providing practical insights for selecting suitable experimental subjects for targeted research. This analysis can offer valuable advantages for future functional research and product development.

MATERIALS AND METHODS

Six types of water kefir grains were used in this study, with HY, GS, LS, SJ, and JW sourced from households in Yongzhou City, Hunan Province, Shenzhen City, Guangdong Province, Shenyang City, Liaoning Province, Jining City, Shandong Province, and Wuxi City, Jiangsu Province, while M339 was purchased from IMMUNRISE Company in the United States as a commercial product.

Activation of kefir grains

10% (w/v) brown sugar aqueous solution was sterilised at 115°C for 10 min. After cooling to room temperature, it was inoculated with 5% (w/v) water kefir grains and incubated in a 30°C incubator for 24 h. After fermentation, kefir grains were filtered through a sterilisation screen, cleaned with sterile normal saline, and activated 3 times.

Determination of fermentability of water kefir grains

The 6 kinds of activated kefir grains were added into sterile brown sugar aqueous solutions at a 2% inoculation rate and cultured in an incubator at 30°C for 48 h. The weight of kefir grains, pH of kefir and lactic acid content were measured every 12 h. After fermentation, the exopolysaccharide yield was measured and a water kefir antibacterial test was conducted.

Water kefir grain growth measurement

The water kefir grains in brown sugar water were filtered out, washed twice with sterile water, and then the wet weight was determined.

Water kefir pH value and acidity measurement

pH was measured using a pH metre. Lactic acid levels were determined by sodium hydroxide titration, and the specific operation methods were as follows: The 100 mL triangular bottle was injected with 1 mL of water kefir, diluted with 9 mL distilled water, and then 2 to 3 drops of 1% phenolphthalein indicator were added. After this, the solution was titrated with 0.l mol/L NaOH standard solution. While titration occurred, the triangular bottle was shaken until a slight red colour appeared, making sure that the colour did not disappear within 30 s of the titration ending.

X – grams of acid per litre of sample, g/L

C – Sodium hydroxide standard titration solution concentration, mol/L

V_1 – the volume of sodium hydroxide standard solution consumed during titration, mL

V_2 – Volume of sodium hydroxide standard solution consumed by the blank group, mL

F – dilution of the solution

K – acid conversion coefficient, lactic acid is 0.09

m – volume of solution, mL.

Determination of extracellular polysaccharide production by water kefir

Glucose standard curve drawing: Weigh 10 mg standard glucose into a 100 ml volumetric bottle, add water to the scale, absorb 0, 0.2, 0.4, 0.6, 0.8, 1.0 ml respectively, and fill with distilled water to 2.0 ml scale. According to Jiang et al. (2021) in the phenol-sulfuric acid method the blank group used 2.0 ml of water with the same operation. The average value was repeated three times to obtain the glucose standard curve, where the horizontal coordinate is the glucose concentration, and the OD value is the ordinate.

Extracellular polysaccharide extraction: The water was inactivated at 100°C for 30 min, centrifuged at 8000 r/min for 20 min, the precipitation was removed, and finally 40% trichloroacetic acid was added to the final concentration of 4% (m/v). Then it was placed at 4°C overnight and centrifuged at 10000 r/min for 30 min. Thereafter, the protein was removed, and then

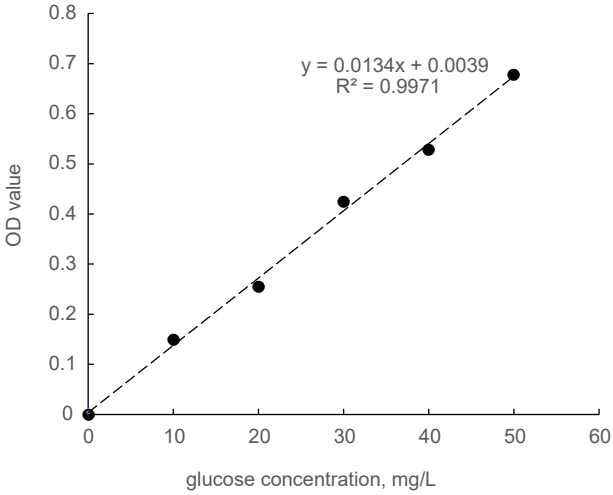


Fig. 1. The standard curve of glucose

95% ethanol was added at 3 times the volume. The polysaccharide was deposited at 4°C overnight and centrifuged at 10000 r/min for 15 min. It was dissolved in sterile water, and a 1 ml sample was measured, in adherence to the phenol-sulfuric acid method.

Water kefir bacteriostatic test

The sterile filter paper with a diameter of 6 mm was soaked in the fermentation broth, and then dried. The air-dried filter paper was placed on the coated indicator bacteria. *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538 were cultured on LB plates for 3 days to observe whether there was a bacteriostatic zone and, if so, measure the diameter of the bacteriostatic zone.

Analysis of water kefir grain metabolites by GC-MS

This analysis was performed according to the method of Zhao (2022) with some modifications. Six kinds of water kefir grains were fermented in brown sugar water under the same conditions (1% m/v concentration of brown sugar water, 5% m/v inoculation amount, fermentation at 30°C for 48 h), compared with sterile and uninoculated sterile brown sugar aqueous solution. After the water kefir fermented for 48 h, the water kefir grains were filtered and centrifuged at 4°C at 14,000 rpm for 10 min to remove a small amount of bacteria in the fermentation solution. The supernatant

was concentrated 10 times, and then methanol/aqueous solution with an equal volume fraction of 80% was added. The metabolites were extracted by swirling for 10 min and centrifuged for 10 min at 4°C at 14,000 rpm. After the supernatant was filtered through the 0.22 μm oil film, 500μL of each supernatant was frozen and dried. The freeze-dried samples were added with 60μL methicillin hydrochloride – pyridine solution (20 mg/mL), swirled until no particles remained, and then were immersed in a constant temperature water bath at 37°C for 90 min. Then 30 μL MSTFA (N-methyl-trimethylsilyl-trifluoroacetamide) was added, mixed evenly with vortex oscillation, and further immersed in a water bath at 37°C for 60 min. The immersed sample was centrifuged at 14000 rpm for 10 min. 75 μL supernatant was taken and placed in the sample bottle for machine analysis.

Gas phase conditions: The column was DB-5MS (30 m × 0.25 mm × 0.25 μm) elastic quartz capillary column, the initial temperature was 70°C, held for 3 min, raised to 200°C at 5°C/min, and then raised to 310°C at 10°C /min, and held for 15 min. The carrier gas was high purity He (99.999%); The front column pressure was 52.538 kPa, and the carrier gas flow rate was 1.0 mL /min. The sample size was 1 μL. Mass spectrum conditions: Ion source was EI source (Agilent 7890/5975C); Ion source temperature 230°C; Quadrupole temperature 150°C; Electron energy 70 eV; Interface temperature 280°C; Quality range 50~800 m/z.

The original data was converted into CDF format using Xcalibur 4.0 software, and then imported into a XC-MS program for noise filtering, retention time alignment, chromatographic peak detection and matching, etc., to obtain a scale. Preliminary qualitative analysis was performed by using standard spectrographic libraries (such as NIST, Fiehn, Wiley, etc.). SIMCA17.0 was used for least squares discriminant analysis (OPLS-DA). T-test was used to screen differential metabolites ($p < 0.05$). Cluster analysis and path analysis were performed by MetaboAnalyst 5.0 software.

RESULTS AND DISCUSSION

Comparison of fermentation properties of water kefir grains

The growth of 6 kinds of aqueous kefir grains in brown sugar water, pH, acidity, EPS yield and antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* are shown in Figure 2 to Figure 3 and Table 1.

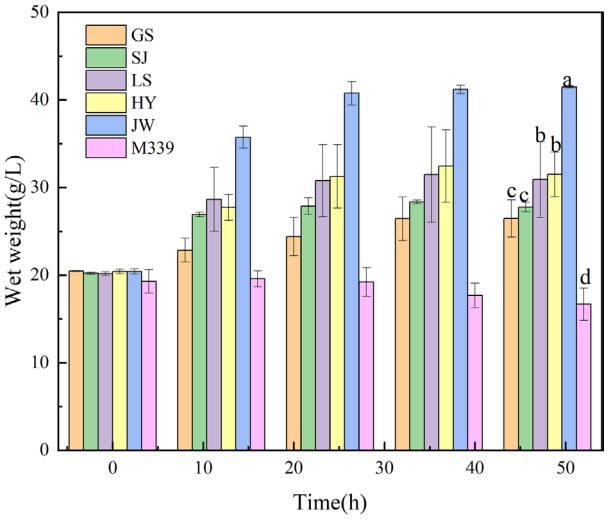


Fig. 2. Changes in the growth of water kefir grains

As shown in Figure 2, with the increase of culture time, the wet weight of five domestic water-kefir grains increased at first, and then tended to be unchanged, with a significant increase compared with the beginning ($p < 0.05$). The fastest growing water kefir grain was JW from Wuxi, Jiangsu Province, whose wet weight increased from 20.40 g/L to 41.47 g/L, an increase of 103%. The slowest growing GS from Shenzhen, Guangdong Province increased its wet weight from 20.46 g/L to 26.46 g/L, an increase of 29%. HY from Yongzhou, Hunan Province, LS from Shenyang, Liaoning Province and SJ from Jining, Shandong Province - the growth rate of these three kinds of water kefir grains was between the levels of JW and GS, with a weight gain of 54%, 53% and 37%, respectively. Literature has shown that water kefir grains can only proliferate on the basis of the original grains, and the proliferation is relatively slow (Azizi et al., 2021). This experiment demonstrates that they do not proliferate well in brown sugar water, aligning with the findings of Cufaoglu and Erdinc (2023). In this experiment, JW was the fastest proliferating, and the wet weight after fermentation was as high as 41.47 g/L. However, American kefir M339 did not grow in brown sugar water, and its wet weight before and after fermentation had no significant change ($p > 0.05$), and its weight even decreased slightly (from

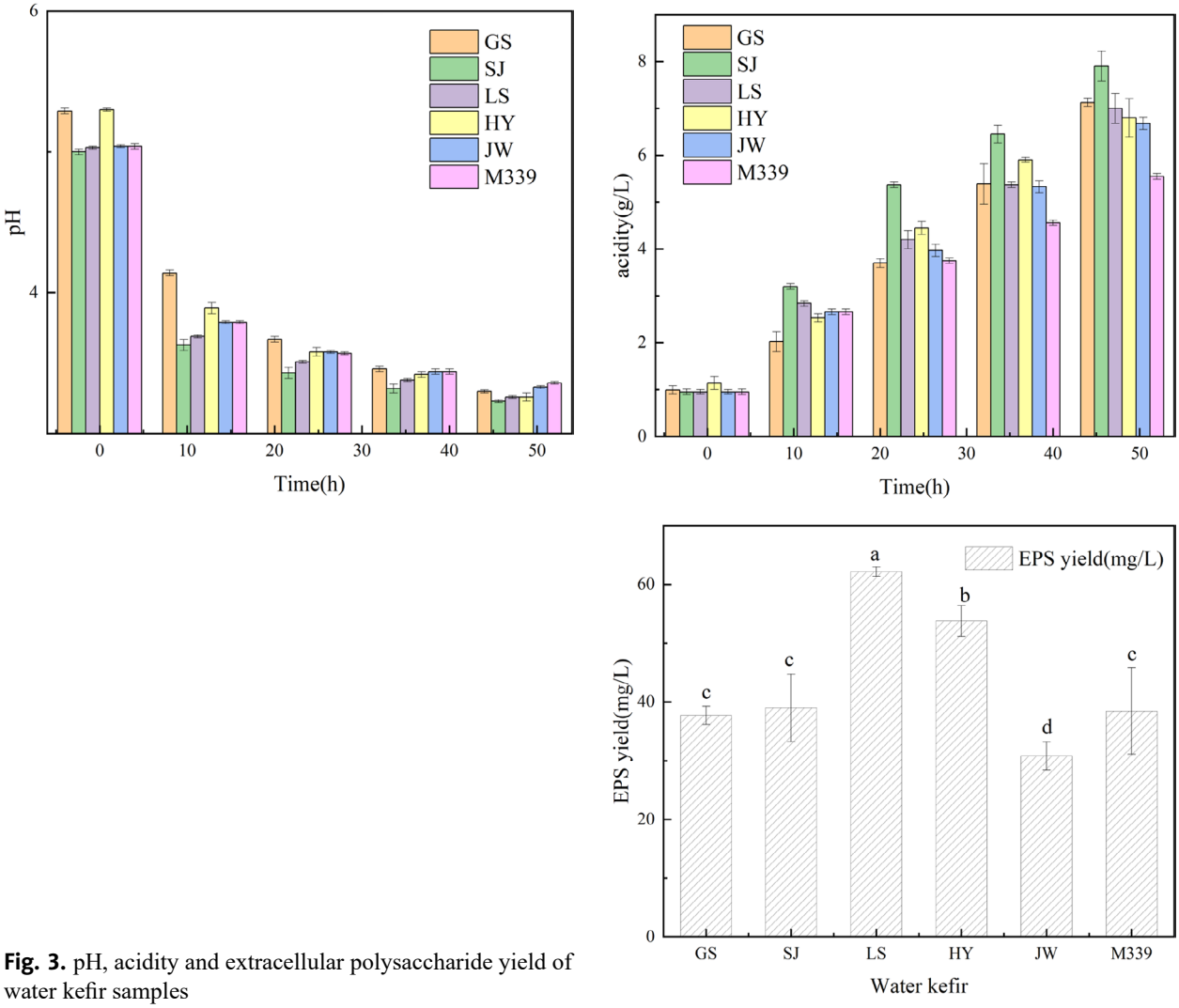


Fig. 3. pH, acidity and extracellular polysaccharide yield of water kefir samples

the initial 19.29 g/L to 16.68 g/L). There has been speculation that freeze-drying may slow the growth of kefir grains, or that the nutrients in brown sugar water may not meet their growth requirements.

As the fermentation progresses, the pH of the water kefir gradually decreases and the acidity (in lactic acid) gradually increases, as shown in Figure 3. Due to the presence of lactic acid bacteria, acetic acid bacteria and other bacteria in water kefir, many organic acids will be produced, thus reducing the pH of the environment (Li et al., 2019). The existence of organic acids can make it have a fresh taste, aroma and a good texture (Van Wyk, 2019). SJ has the highest acid-producing capacity, with pH dropping from 5 to 3.23 and an acidity (in lactic acid) of 7.90 g/L, with acidity increasing by 6.95 g/L before and after fermentation. Destro et al. (2019) used water kefir grains to ferment a common brown sugar solution, with the pH being at 3.77 and the amount of lactic acid was 1.58 g/L at 25°C and 48 h. In contrast, the temperature in this study is more suitable for the growth of acid-producing lactic acid bacteria, so the pH is lower and the acidity is higher. In the six kinds of water kefir, the ability to produce exopolysaccharides was as follows: LS > HY > SJ > M339 > GS > JW, with the highest and lowest values of 62.16 mg/L and 30.82 mg/L respectively. Its exopolysaccharide is considered to be a new and safe potential source for food and functional materials

Table 1. Antibacterial effect of six kinds of water kefir

Inhibition zone diameter mm	GS	SJ	LS	HY	JW	M339
E. coli	8.50 ±0.50^{d}	13.67 ±1.53^{a}	8.25 ±0.35^{d}	8.50 ±0.87^{d}	7.75 ±0.35^{e}	6.00 ±0.00^{f}
S. aureus	10.47 ±0.84^{b}	9.25 ±0.35^{c}	8.50 ±0.71^{d}	9.75 ±1.50^{c}	9.17 ±0.76^{c}	9.50 ±0.71^{c}

with a variety of physiological activities. The different sources of kefir grains, fermentation substrate and culture conditions will affect the fermentation characteristics of water kefir. Dong Xinxin et al. (2022) fermented Cherry Tomato juice with water kefir grains. At 18 h of fermentation, the pH was 3.75, the acidity was 18.40 g/L, and the weight gain ratio of water kefir grains was 0.20.

In Table 1, different sources of water kefir showed different antibacterial abilities, and the differences were significant ($p < 0.05$). Water Kefir SJ had the strongest inhibition on *Escherichia coli*, and its inhibition zone was 13.67 mm. GS had the strongest inhibitory activity against *Staphylococcus aureus*, and its inhibitory zone was 10.47 mm. SJ has strong bacteriostatic ability against the two pathogens, and the acid production ability of SJ is also strong as measured above, which may be because the organic acids contained in water kefir are a class of bacteriostatic substances. These can inhibit the growth of pathogens (Gamba et al., 2019). However, SJ has a stronger inhibition ability on *Escherichia coli* than *Staphylococcus aureus*, which may be caused by the synergistic effect of organic acids, hydrogen peroxide, bacteria and other antibacterial substances (Gut et al., 2021). In addition, various literature has shown that kefir grains also have certain antibacterial effects (Kakisu et al., 2007).

Analysis of metabolites in six kinds of water kefir

The kefir beverages were analysed by gas chromatography-mass spectrometry (GC-MS) after particle removal, centrifugation, concentration and derivatization. The collected metabolic data will be analysed and processed.

The detected metabolites were searched through mass spectral database (NIST, etc.), and 131 metabolites were identified, as shown in Figure 4. It includes 22 kinds of sugars, 35 kinds of esters, 29 kinds of acids, 10 kinds of alcohols, 8 kinds of nucleosides, 6 kinds

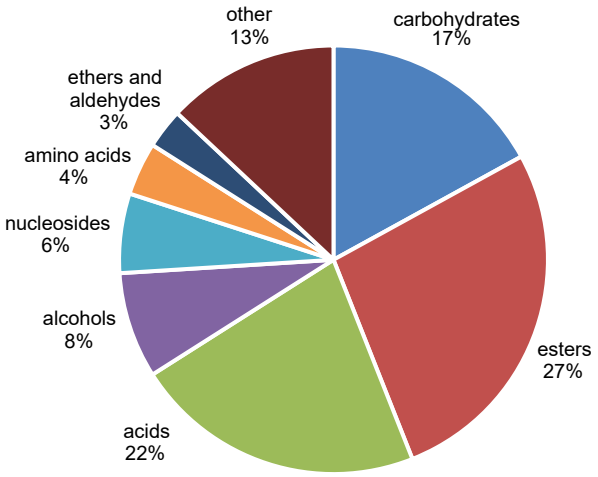


Fig. 4. Percentage distribution of metabolites

of amino acids, 4 kinds of ethers and 17 kinds of organic compounds. There are isomaltose, D-glucose, L- (+) -arabinose, cellobiose and so on. Acids include lactic acid, isocitric acid, malonic acid, etc. As one of the main organic acids in kefir, lactic acid may contribute to the activity of other antibacterial metabolites in kefir and destroy target cells through the permeability of the cell membrane (Sadeghi et al., 2018). Alcohols include glycerol, D-mannitol, xylitol, etc. Amino acids are L-valine, L-glutamic acid, L-proline and so on. In addition, studies have shown that amino acids produced by kefir fermentation are positively correlated with organic acids in the correlation network, showing a collaborative relationship that can promote the positive interaction between these elements (Ma et al., 2024).

After pretreatment, such as peak area normalisation and missing value filling, the obtained metabolic data were imported into SIMCA17 software for partial least square discriminant analysis (PLS-DA), as shown in Figure 5.

After the samples are analysed, there are relative coordinate points on the graph, and the spacing of

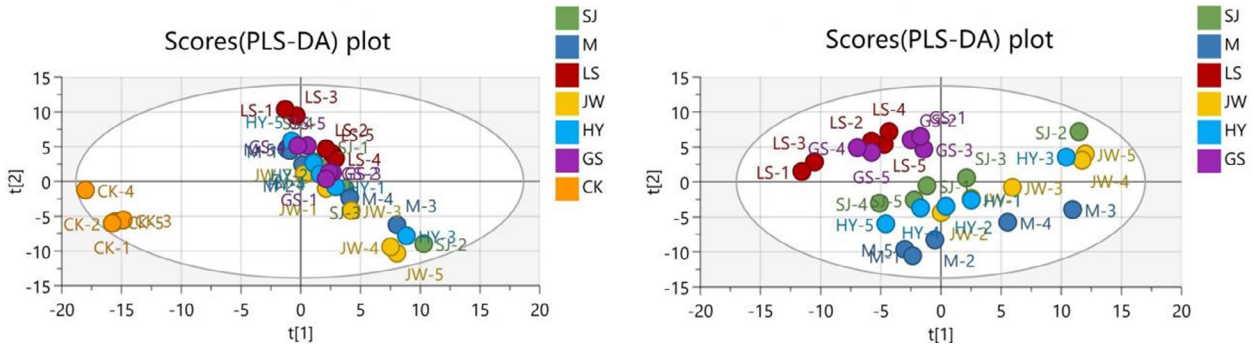
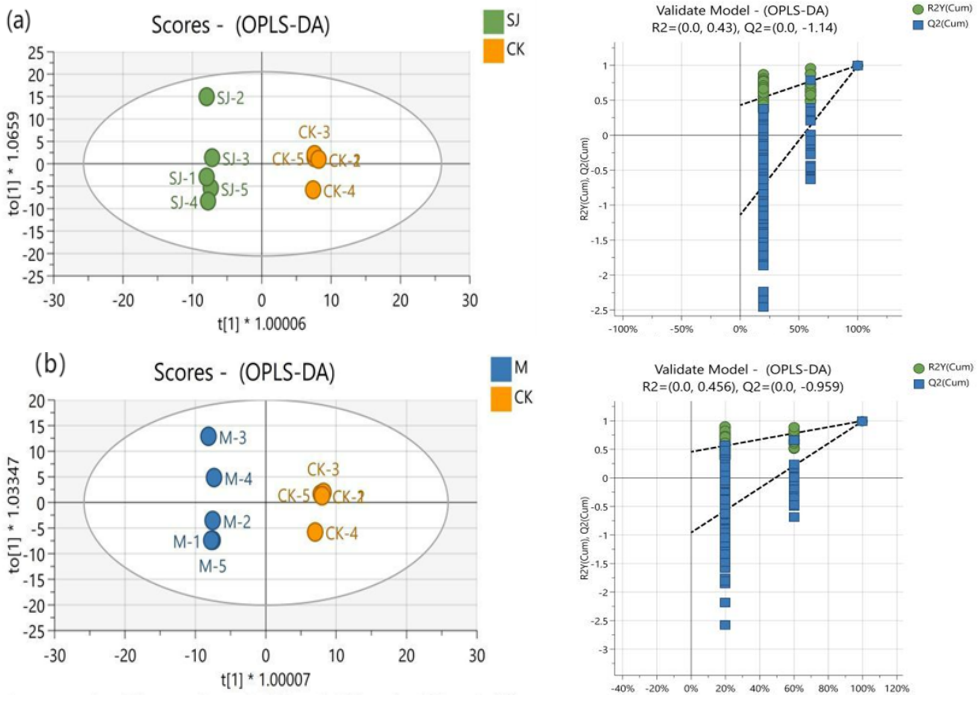


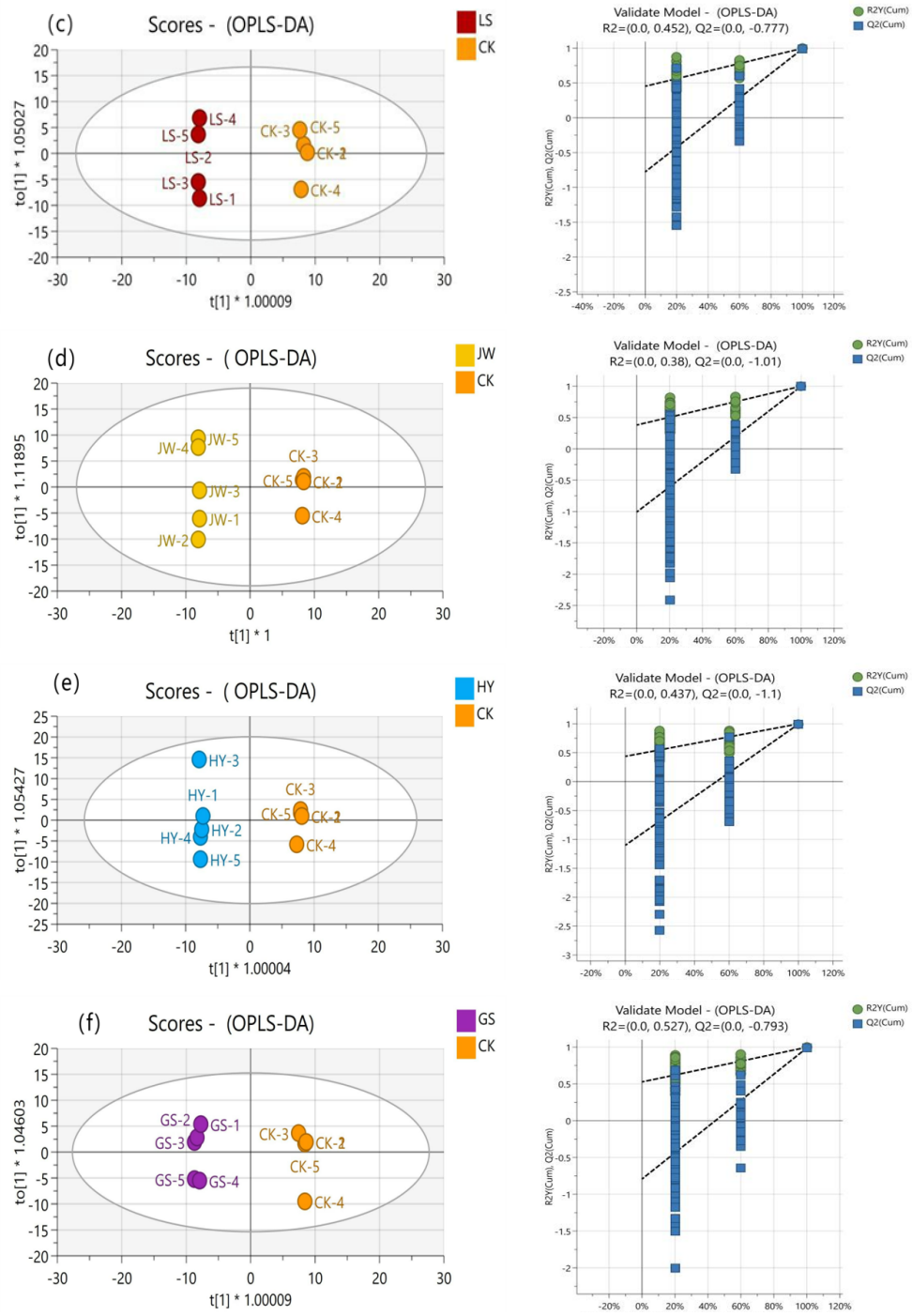
Fig. 5. The PLS-DA score plots of water kefir samples

each coordinate represents the degree of aggregation and dispersion among samples. The different water kefir samples were all within the 95% confidence interval, indicating no difference in the samples. All kinds of distribution in this group are relatively clustered, and the parallelism is good. CK (control group) and other samples showed an obvious tendency to separate, which was related to the metabolites produced by microorganisms during the fermentation of kefir grains.

Analysis of different metabolites of six kinds of water kefir

Orthogonal partial least squares discriminant analysis (OPLS-DA) corrects PLS-DA model by orthogonal transformation, which can distinguish the difference between groups better and improve the effectiveness and resolution of the model. In order to verify whether the OPLS-DA model is overfitting, a replacement test randomly grouped for 200 times is usually set, and the results are shown in Figure 6.





R^2Y and Q^2 represent the explanatory and predictive power of the model. The closer R^2Y and Q^2 are to 1, the more stable and reliable the model is and the higher its predictive power will be. In SJ and CK, R^2Y=0.998, Q^2 = 0.993; In M and CK, R^2Y = 0.998, Q^2 = 0.994; In LS and CK, R^2Y = 0.998, Q^2 = 0.991; In JW and CK, R^2Y = 1, Q^2 = 0.997; In HY and CK, R^2Y = 0.999, Q^2 = 0.994; In GS and CK, R^2Y = 0.997, Q^2 = 0.988. Therefore, the model established in this study is stable and reliable and can better distinguish the differences between groups.

Fig. 6. The OPLS-DA score chart and permutation testing chart: a – SJ vs CK; b – M vs CK; c – LS vs CK; d – JW vs CK; e – HY vs CK; f – GS vs CK)

According to the weighting coefficient of the OPLS-DA model, the variables with high contribution to the model were screened. VIP scores (VIP >1, *p*--value < 0.05) and the differential metabolites between groups were obtained. Sorted by VIP value, the top ten differential metabolites of VIP value in each group were selected, and the results were shown in Tables 2, 3, 4, 5, 6 and 7.

Among the six groups of differential metabolites, there were significant differences between the groups, and we can see a pattern in that the differences were the same between the groups, such as D-threitol and 3-phenyllactic acid. There are also major differences, such as putrescine, uracil, L-glutamic acid and so on. Volcanic maps of these six groups of differentiated metabolites are shown in Figure 7.

Table 2. Differential metabolites of SJ and CK

Metabolite	Retention time	VIP value
D-threitol	20.7208	1.3695
Butanedioic acid, methyl-, bis(trimethylsilyl) ester	15.9125	1.3706
Propanoic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester	10.5250	1.3805
Butane, 2,3-bis(trimethylsiloxy)-	6.6583	1.3594
3-phenyllactic acid	22.4833	1.3525
Uracil	16.1625	1.3519
3,6-Dioxa-2,7-disilaoctane, 2,2,4,7,7-pentamethyl-	6.9750	1.3433
Pentasiloxane, dodecamethyl-	11.2208	1.3422
4-Hydroxyphenylethanol, di-TMS	22.2375	1.3575
2-Hydroxyisovaleric acid	11.0625	1.3416

Table 3. Differential metabolites of M and CK

Metabolite	Retention time	VIP value
Butane, 2,3-bis(trimethylsiloxy)-	6.6583	1.3755
Propanoic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester	10.5250	1.3701
Pentasiloxane, dodecamethyl-	11.2208	1.3662
D-threitol	20.7208	1.3626
4-Hydroxyphenylethanol, di-TMS	22.2375	1.3559
3,6-Dioxa-2,7-disilaoctane, 2,2,4,7,7-pentamethyl-	6.9750	1.3542
L-glutamic acid (dehydrated)	20.9375	1.3510
Ethyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-D-glucopyranoside	27.9542	1.3507
Silane, trimethyl(2-phenylethoxy)-	12.9458	1.3518
2-Hydroxyisovaleric acid	11.0625	1.3488

Table 4. Differential metabolites of LS and CK

Metabolite	Retention time	VIP value
Propanoic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester	10.5250	1.3105
Butanedioic acid, methyl-, bis(trimethylsilyl) ester	15.9125	1.3037
D-glucose	29.7542	1.2996
Ethyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-D-glucopyranoside	27.9542	1.2971
Aspartic acid	18.5417	1.2969
Butane, 2,3-bis(trimethylsiloxy)-	6.6583	1.2967
Butanoic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester	11	1.2967
4-Hydroxyphenylethanol, di-TMS	22.2375	1.2965
D-threitol	20.7208	1.2943
3-phenyllactic acid	22.4833	1.2931

Table 5. Differential metabolites of JW and CK

Metabolite	Retention time	VIP value
Propanoic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester	10.5250	1.3099
Butane, 2,3-bis(trimethylsiloxy)-	6.6583	1.3098
4-Hydroxyphenylethanol, di-TMS	22.2375	1.3025
D-threitol	20.7208	1.3024
3-phenyllactic acid	22.4833	1.2959
Butanedioic acid, methyl-, bis(trimethylsilyl) ester	15.9125	1.2930
Pentasiloxane, dodecamethyl-	11.2208	1.2914
2-Hydroxyisovaleric acid	11.0625	1.2910
3,6-Dioxa-2,7-disilaoctane, 2,2,4,7,7-pentamethyl-	6.9750	1.2841
3,4-Dimethoxyphenol, trimethylsilyl ether	19.7583	1.2833

The horizontal coordinate shows the multiple change value of the difference in metabolite content between the two groups, that is, log2FC, and the point off centre indicates the larger difference multiple. The ordinate is the statistical test value of the difference in metabolite content, that is, -log10(*p*-value). The larger the value is, the more significant the difference in metabolite content is. Each point in the diagram represents a specific metabolite. The point on the left (blue) is the metabolite whose content is decreasing, and the point on the right (red) is the metabolite whose content is increasing, and the difference between the two points is more significant. Through analysis, it was found that the substances with increased concentration were mostly acids, esters, alcohols, etc., while the substances with decreased concentration were mostly sugars.

On this basis, the top 25 different compounds with great influence on the model were selected for hierarchical clustering (HCA) analysis, and the results

Table 6. Differential metabolites of HY and CK

Metabolite	Retention time	VIP value
Propanoic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester	10.5250	1.3774
Butane, 2,3-bis(trimethylsiloxy)-	6.6583	1.3710
D-threitol	20.7208	1.3667
Butanedioic acid, methyl-, bis(trimethylsilyl) ester	15.9125	1.3653
4-Hydroxyphenylethanol, di-TMS	22.2375	1.3630
2-Hydroxyisovaleric acid	11.0625	1.3588
3-phenyllactic acid	22.4833	1.3580
D-glucose	29.7542	1.3536
Pentasiloxane, dodecamethyl-	11.2208	1.3533
3,6-Dioxa-2,7-disilaoctane, 2,2,4,7,7-pentamethyl-	6.9750	1.3472

Table 7. Differential metabolites of GS and CK

Metabolite	Retention time	VIP value
Propanoic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester	10.5250	1.2881
Butanedioic acid, methyl-, bis(trimethylsilyl) ester	15.9125	1.2817
3-phenyllactic acid	22.4833	1.2812
Butane, 2,3-bis(trimethylsiloxy)-	6.6583	1.2807
D-glucose	29.7542	1.2804
4-Hydroxyphenylethanol, di-TMS	22.2375	1.2783
D-threitol	20.7208	1.2762
Ethyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-D-glucopyranoside	27.9542	1.2746
Putrescine	25.9833	1.2739
l-Leucine, trimethylsilyl ester	10.8542	1.2704

are shown in Figure 8. In Figure 8, the metabolite clustering tree is shown on the left. The closer the branches are, the closer the expression patterns of all metabolites in the sample.. The branches in the upper tree represent the differences between groups, and the closer the branches are, the smaller the differences between groups. Each column represents a sample, and the sample name is shown below; each row represents a metabolite, the colour block represents the relative expression amount of the metabolite in the sample, the colour represents the relative expression amount of the metabolite in the sample, and the gradient colour block shows the corresponding relationship between the colour gradient and the value. It can be seen from the figure that there are a lot of sugars and more amino acids in the brown sugar aqueous solution of group CK, such as leucrose, Raffinose, D-glucose, D-(+) trehalose, L-glutamic acid (dehydrated), aspartic acid, etc. After water kefir fermentation, GS, HY, JW, LS, M and SJ groups produced abundant acids, alcohols and esters,

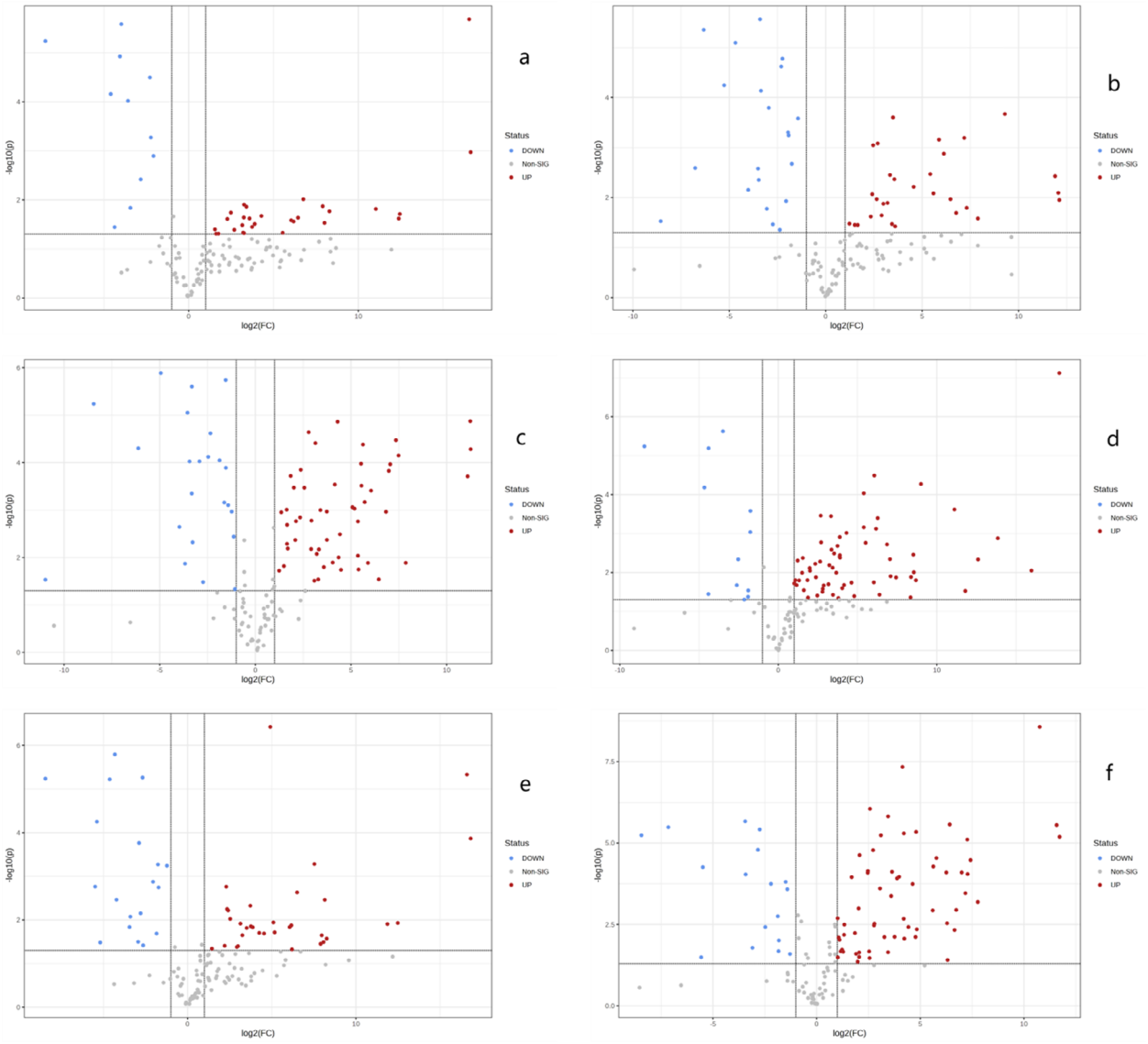


Fig. 7. The volcanic map of differential metabolites: a–f – SJ, M, LS, JW, HY, GS vs CK

and their differences were significant. GS produces more 2,3,4,5,6-pentakis-O-(trimethylsilyl)- D-Glucose, L-glutamic acid and L-Leucine, trimethylsilyl ester; in JW, D-(+)-melezitose, 2,2,8,8-tetramethyl-3,7-Dioxa-2,8-disilanonane and Propanoic acid, The content of 3-[(trimethylsilyl)oxy]-trrimethylsilyl ester is high; LS produced more isocitric acid, citric acid, Ethyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-D-glucopyranoside; in M, 2,3-bis(trimethylsiloxy)-Butane, Butanoic acid, 4-[(trimethylsilyl)oxy]-trimethylsilyl ester, 2,2,4,7,7-pentamethyl-3,6-Dioxa-2,7-disilaoctane content is high.

Use box Figures 9 to more intuitively compare the relative content differences of 14 key metabolites from 6 different water kefir.

In Figure 9, green, red, blue, purple and yellow represent sugars, organic acids, amino acids, alcohols and esters, respectively. Compared with the CK

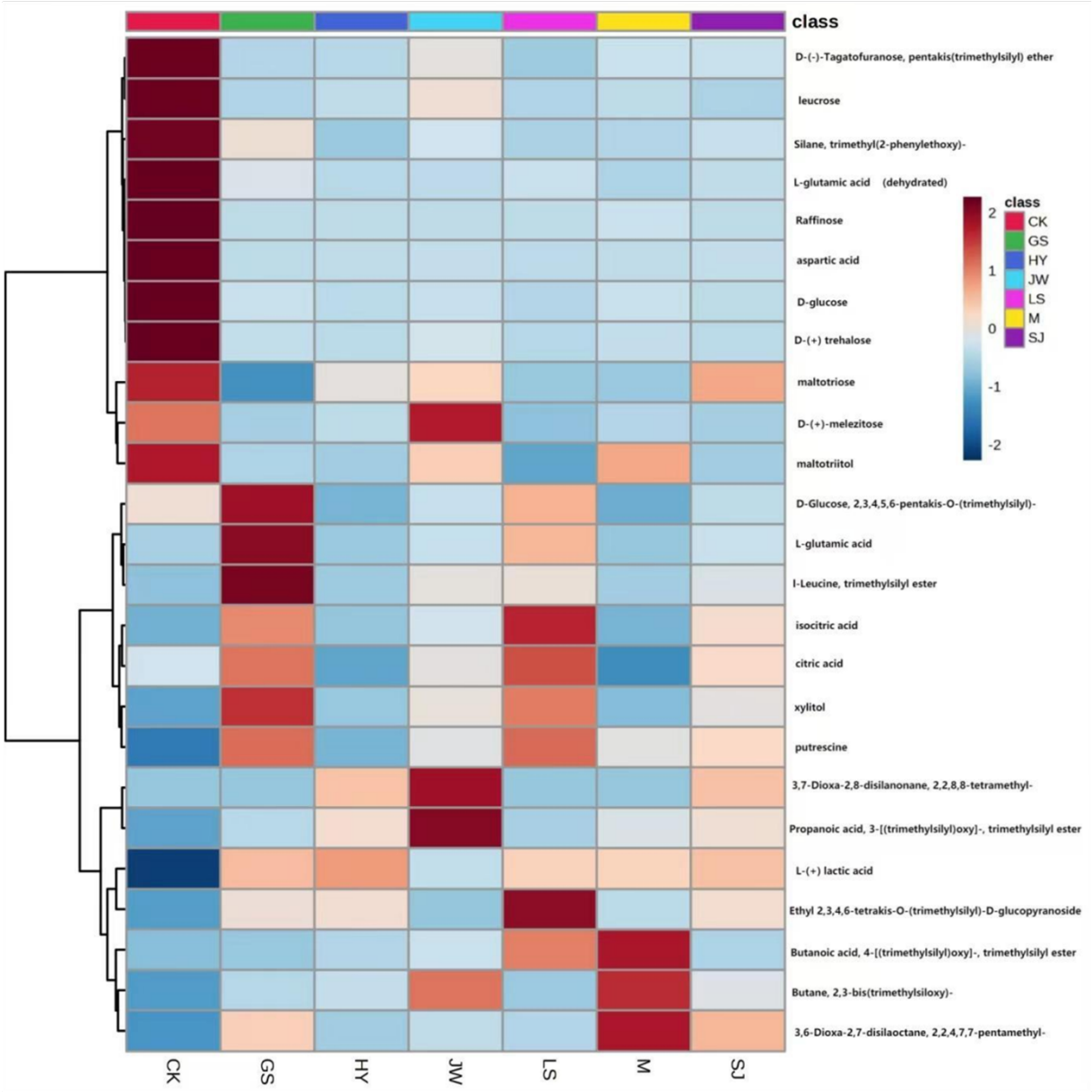


Fig. 8. The heat map of differential metabolites

group, it can be found that the glucose content was significantly reduced after fermentation, which was basically consumed by the microorganisms in the kefir grains. Part of the reason may be that the yeast used it as a substrate more efficiently (Destro et al., 2019). Moreover, Laureys et al. (2014) observed that glucose levels decreased to near zero after 48 hours of fermentation. Additionally, maltotriose levels also decreased, but the content of maltotriose in each water kefir varied significantly. The concentration of maltotriose was the lowest in GS, higher in M399 and LS, and higher in the other three. The results indicated that there were significant differences in the utilisation rate of maltotriose in different kefir grains. The

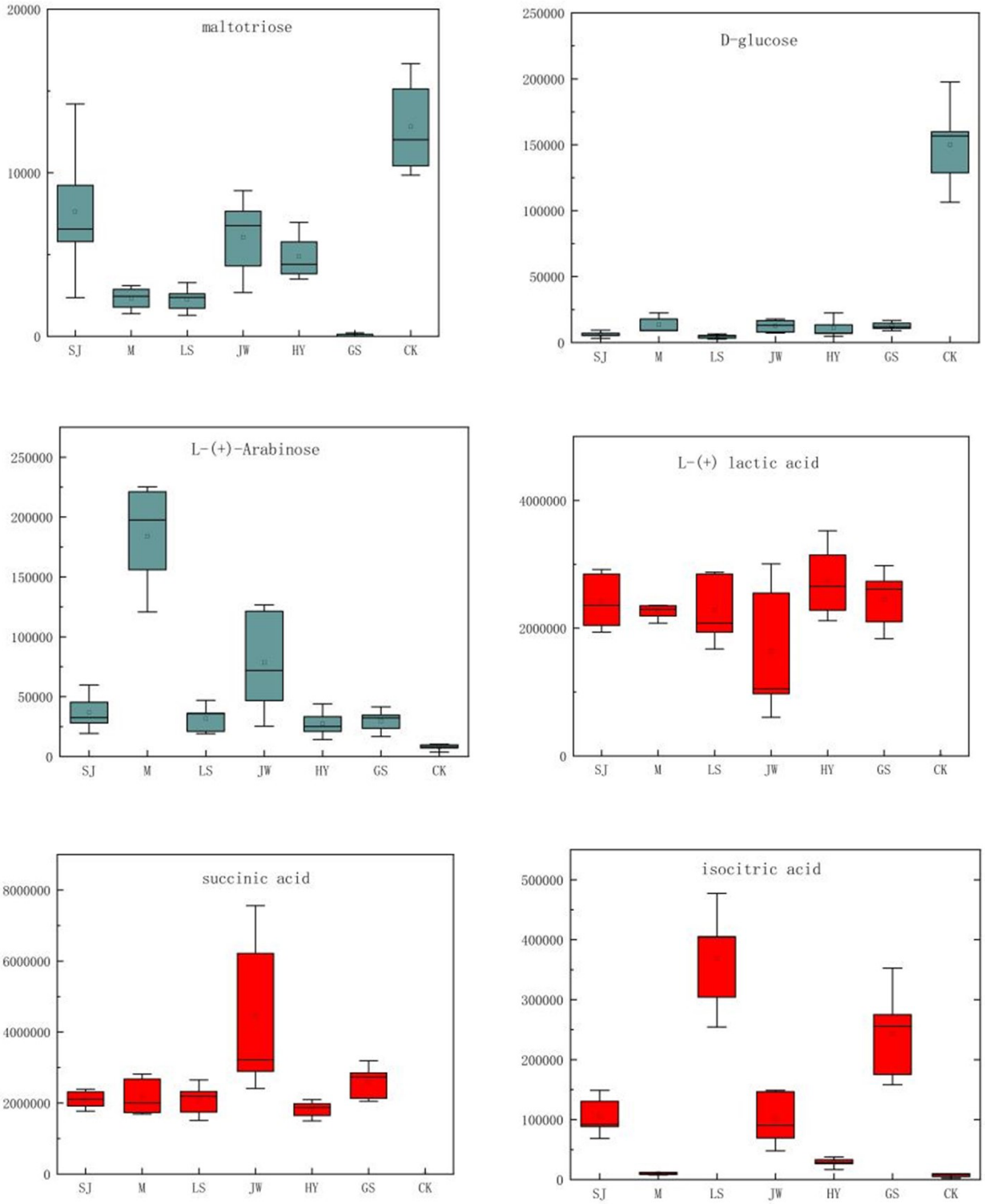


Fig. 9. Variation of relative abundance of different compounds in water kefir

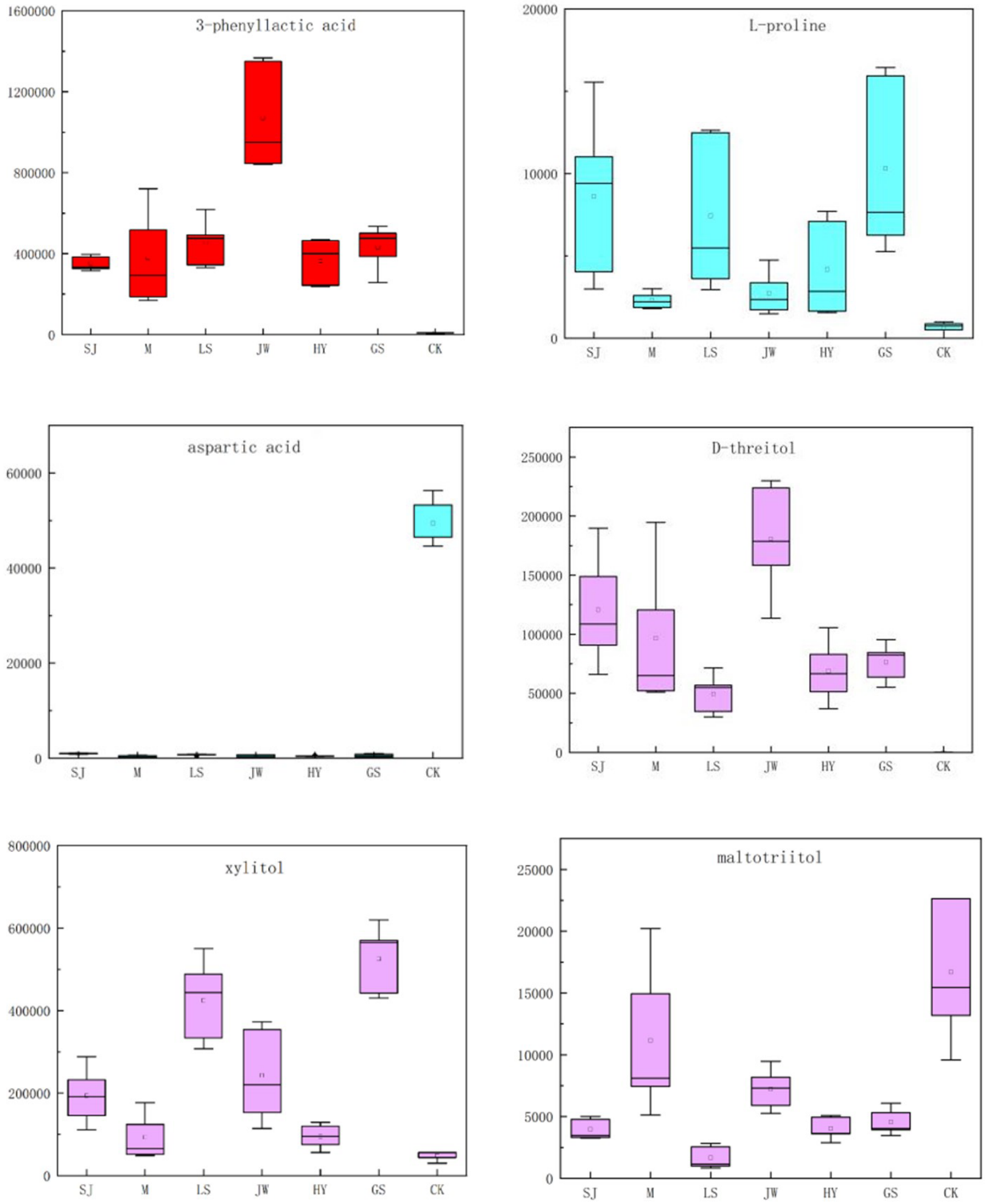

3-phenyllactic acid
1600000
1200000
800000
400000
0
SJ
M
LS
JW
HY
GS
CK
L-proline
20000
10000
0
aspartic acid
60000
40000
20000
0
D-threitol
250000
200000
150000
100000
50000
xylitol
800000
600000
400000
200000
maltotriitol
25000
20000
15000
10000
5000

Fig. 9. – cont.

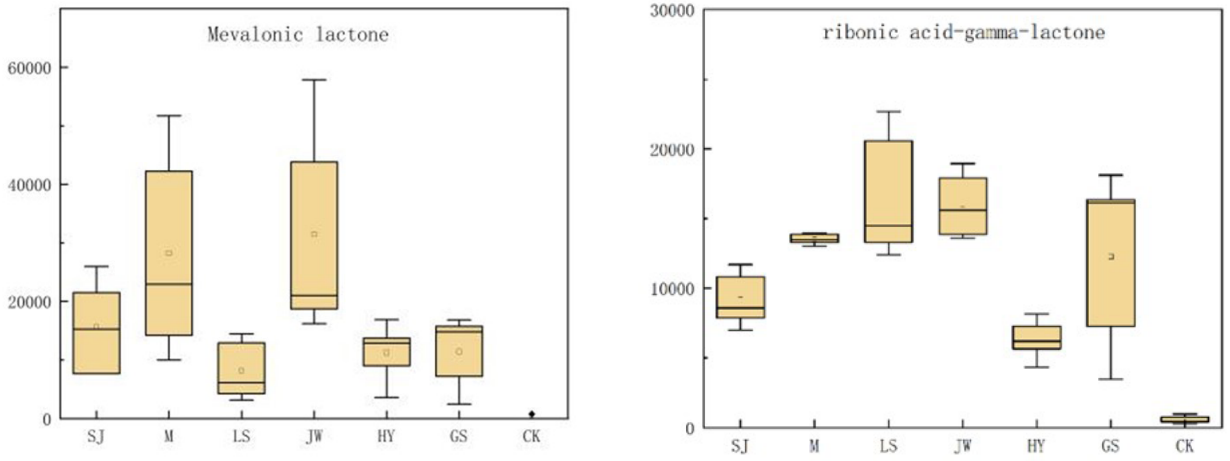


Fig. 9. – cont.

content of L-(+) -Arabinose increased significantly, and the highest was found in M399, followed by JW, and the other 4 were lower. This shows that the bacteria in water kefir use the sugar substances in the brown sugar water to participate in their own metabolism, and in this process, they will synthesise new sugar substances. Studies have shown that L-arabinose can reduce the weight of rats and regulate the blood sugar level, which has certain effects on weight loss and the level of blood sugar (Chen, 2022). Alsayadi et al. (2014) showed that long-term (35 days) feeding of 10–30% kefir reduced blood glucose by 100–200 g/dL in streptozotocin-induced diabetic rats, and improved their body weight and blood lipids. This is likely due to the large amount of arabinose produced by kefir fermentation.

The fermentation of brown sugar water by water kefir grains results in the production of a diverse array of organic acids, including lactic acid, succinic acid, isocitric acid, and other acids. The amount of the same organic acid produced by different water kefir is not the same. Lactic acid exists in the metabolites of these six kinds of water kefir, which will reduce the environmental pH and inhibit the growth of microorganisms such as *Escherichia coli* (Gao and Zheng-jun, 2014). JW has a strong ability to produce succinic acid, which is an effective antioxidant and an intermediate in the tricarboxylic acid cycle (TCA) and plays an important role in the process of mitochondrial ATP production, as well as regulating intestinal inflammatory response and body immune function (Macias-Ceja et al., 2019; Nadjsombati et al., 2018). In the figure, the isocitric acid content of LS is the largest, while the content of M and HY is relatively small. Isocitric acid is a key substance in the tricarboxylic acid cycle and its supplement reaction, and plays an important role in the energy metabolism, synthesis and antioxidant process of organisms (Morgunov et al., 2018). Compared with CK group, the content of 3-phenyllactic acid significantly increased, and the content of JW was the highest. Because 3-phenyllactic acid has a broad-spectrum antibacterial ability, and has a synergistic effect with other antibacterial agents, it can be applied broadly in food preservation (Li et al., 2022).

During the fermentation process, microorganisms in water kefir grains use aspartic acid in the fermentation substrate as a nitrogen source to synthesize new amino acids, such as L-proline, which can scavenge reactive oxygen species (ROS) (Meena et al., 2019). In addition to amino acids, there are many alcohols in water kefir. The alcohol production capacity of kefir grains in different water varies. The content of D-threitol was the highest in JW, followed by SJ and M, and the lowest in LS. The content of xylitol in GS was the highest, while the content of M and HY was relatively small. Xylitol is an intermediate in carbohydrate metabolism and has many probiotic functions, including reducing blood sugar level and preventing dental

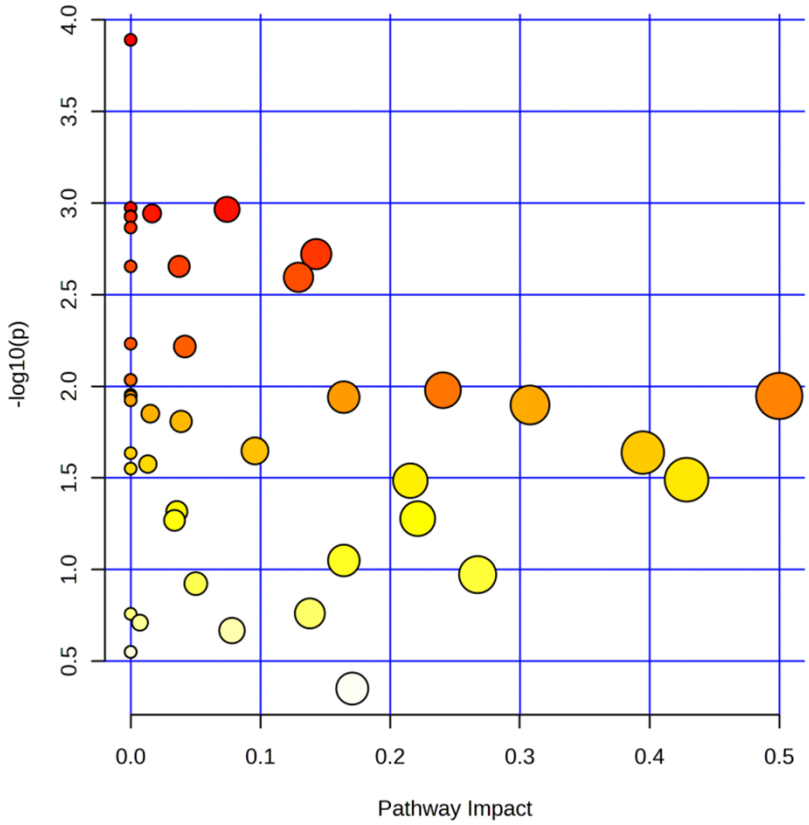


Fig. 10. Enrichment analysis of metabolic pathways

cavities (Gasmi Benahmed et al., 2020). The content of maltotriitol was the highest in CK group, followed by M and JW, and the lowest in LS group. Because of the concentration treatment in the early stage, there are no aromatic components in the metabolites measured, and the ester substances are mostly derivatives. JW and M produced more mevalonic lactone, while LS produced less. In the presence of water, mevalonic lactone rapidly converts to mevalonic acid, which is an important intermediate in the synthesis of molecules such as cholesterol (Esen et al., 2022). The production of ribonic acid-gamma-lactone was significantly different among the six kinds of water kefir, with the highest LS and the lowest HY.

In order to further understand the differences in metabolic pathways between different water kefir, MetaboAnalyst 4.0 was used to analyse metabolic pathways, and the results are shown in Figure 10.

In bubble Figure 10, all matched paths are displayed according to the *p*-value in path enrichment analysis and the influence value of paths in path topology analysis. The ordinate -Log(p) value is obtained from the path enrichment analysis, and the larger the -Log(p) value, the redder the colour (the smaller the *p*-value); The x-coordinate Pathway Impact value was obtained from the path topology analysis, and the larger the Pathway Impact value, the larger the bubble. The greater the -Log(p), the more obvious the difference, and the larger the Pathway Impact value, the greater the role of metabolites in the pathway. Hence, the pathway in the upper left is the most significant. The six water kefir compounds were mainly related to valine, leucine and isoleucine degradation, nitrogen metabolism, pyrimidine metabolism, purine metabolism, TCA cycle, amino sugar and nucleotide sugar metabolism.

CONCLUSION

The wet weight, pH value, acidity of fermentation solution, exopolysaccharide yield and antibacterial activity of 6 types of water kefir grains were determined. The findings revealed varying fermentation characteristics among the different sources of water kefir grains. Specifically, JW exhibited the fastest increase, SJ produced more acid, its fermentation liquid showed the strongest inhibition effect on *Escherichia coli*, and LS had the highest exopolysaccharide yield.

The kefir metabolites from 6 different sources of water kefir beverage were analysed and detected by gas chromatography-mass spectrometry (GC-MS). A total of 131 metabolites were identified, including 22 kinds of carbohydrates, 35 kinds of esters, 29 kinds of acids, 10 kinds of alcohols, 8 kinds of nucleosides, 6 kinds of amino acids, 4 kinds of ethers and aldehydes, and 17 kinds of other organic compounds. Orthogonal partial least squares discriminant analysis (OPLS-DA) found significant differences between different water kefir groups. Sugar and aspartic acid in brown sugar water were fermented by six kinds of kefir grains to produce acids, alcohols and esters. The contents of L-proline and xylitol in water kefir GS, D-threitol, succinic acid and 3-phenyllactic acid in JW, isocitric acid in LS and L-arabinose in M were significantly higher than those in other water kefir. The production of these substances provides the water kefir grains with their unique flavour and prebiotic properties. Therefore, identifying the differences of water kefir grains from different sources is conducive to targeted development and functional research.

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DECLARATIONS

Data statement

All data supporting this study has been included in this manuscript.

Ethical Approval

Not applicable.

Competing Interests

The authors declare that they have no conflicts of interest.

OPEN ACCESS

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