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Effects of Fermentation Period on the Non-Volatile Metabolites of Chinese Ultra-Long-Term Solid Fermented Kohlrabi Based on Non-Targeted Metabolomic Analysis

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Abstract: This study aimed to investigate the effects of ultra-long-term fermentation on the formation of non-volatile metabolites of Chinese solid-fermented kohlrabies. Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) based non-targeted metabolomics coupled with multivariate statistical analysis were employed to respectively analyze the kohlrabies solid fermented for 5 years (5Y), 8 years (8Y), and 11 years (11Y). The results showed that 31, 169, and 123 differential metabolites were identified in the three groups of 5Y and 8Y (A1), 5Y and 11Y (A2), and 8Y and 11Y (A3), respectively (VIP > 1, $p < 0.05$ and $|\log_2 FC| > 1$). The differential non-volatile metabolites were mainly organic acids and derivatives, organoheterocyclic compounds, benzenoids, lipids and lipid-like molecules, and organicoxygen compounds. Furthermore, 11 common differential metabolites were screened in the three groups, including diaminopimelic acid, ectoine, 9,10,13-TriHOME, and 9 others. The citrate cycle, glycine, serine and threonine metabolism, pantothenate and CoA biosynthesis, and glyoxylate and dicarboxylate metabolism were the four pathways most significantly correlated with the differential non-volatile metabolites based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis ($p < 0.05$). The present study describes the effects of ultra-long-term fermentation periods on the formation of non-volatile metabolites in solid fermented kohlrabies, providing a theoretical basis for cooking with the three solid fermented kohlrabies to make different Chinese dishes.

Keywords: kohlrabi; metabolomics; LC-MS/MS; differential metabolites; metabolic pathway

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1. Introduction

Kohlrabi (*Brassica juncea* var. *megarrhiza* Tsen et Lee), belonging to the cruciferous family, is mainly grown in Sichuan, Guangxi, Jiangsu, and Henan provinces in China [1]. The national production of kohlrabi in 2017 was approximately 7,998,000 tons, according to the cultivated area [2]. Kohlrabi is rich in proteins, vitamins, minerals, and many other bioactive components, which is also an excellent source of carotenoids and glucosinolates, exhibiting good nutritional and medical values [3–5]. However, raw kohlrabi typically has a strong mustard spicy taste, which will become crisp and tender, with a rich aroma and a delicious taste after solid fermentation [6]. Fermentation is the commonly used strategy to improve the nutritional value and organoleptic properties of foods by converting organic compounds [7]. In China, solid fermented kohlrabi is usually used to prepare side dishes. Moreover, the solid fermented kohlrabi can also be used, as an ingredient, to cook many kinds of favorite dishes in China [8].

Currently, researches on kohlrabi are mainly focused on flavor components [9], nutritional functions [10], and microbial diversity [11]. For example, the volatile flavor substances of low-temperature and low-salt solid-fermented Chinese kohlrabi (LSCK) and traditional high-salt solid-fermented Chinese kohlrabi (HSCK) have been investigated [12]. Sixteen volatile flavor substances were identified, which could be used as the potential biomarkers to distinguish the two different kinds of solid fermented kohlrabies. It has been reported that

kohlrabi is rich in serotonin and melatonin, which contribute to preventing cardiovascular diseases such as obesity and dyslipidemia [13]. On the other hand, previous studies suggested that lactic acid bacteria, including *Enterococcus*, *Streptococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus* [14], were the dominant bacteria in kohlrabi fermentation.

Normally, the kohlrabies fermented for 3–5 months are used to prepare side dishes in China. However, the 5Y and 8Y kohlrabi are suitable for cooking braised dishes and sour soup, respectively. In contrast, the 11Y kohlrabi is usually used for making fresh soup. Metabolites are essential factors affecting the flavor of fermented foods. However, the effects of ultra-long-term solid fermentation on the formation of non-volatile metabolites in kohlrabi still remain unclear. In this study, the non-volatile metabolites of the 5Y, 8Y, and 11Y solid fermented kohlrabi were analyzed by LC-MS/MS-based non-targeted metabolomics technology. The differential metabolites were subsequently screened by multivariate statistical analysis such as principal component analysis (PCA) and orthogonal partial least squares discrimination analysis (OPLS-DA). Furthermore, the metabolic pathways were enriched by the KEGG database to better analyze the possible pathways significantly correlated with the differential metabolite biosynthesis. This study reveals the effect of ultra-long-term fermentation on the formation of non-volatile metabolites in Chinese solid-fermented kohlrabi, providing a theoretical basis for developing different dishes with ultra-long-term solid-fermented kohlrabi used as an ingredient.

2. Materials and Methods

2.1. Production of Ultra-Long-Term Solid Fermented Kohlrabi

The ultra-long-term solid fermented kohlrabies were produced according to our previous study [15]. The raw kohlrabies were washed with water and subsequently air-dried outside for about 30 days at an average temperature of 7–12 °C. Then, the air-dried kohlrabies were transferred into 200 kg-fermentation tanks. Eight percent (*w*/*w*) salt (upper layer 60%, middle layer 30%, and lower layer 10%) was added to the kohlrabies, followed by 5 days of pickling. Then, 5% (*w*/*w*) salt (upper layer 10%, middle layer 30%, and lower layer 60%) was added to the pickled kohlrabies, followed by 4 days of pickling. Finally, the pickled kohlrabies were respectively fermented for 5 years (5Y), 8 years (8Y), and 11 years (11Y) at room temperature.

2.2. Extraction of Metabolites from Kohlrabi

Extraction of metabolites from 5Y, 8Y, and 11Y kohlrabi was performed as described in our previous study [16]. Kohlrabi (50 mg) was transferred into a 2 mL microtube, and a 6 mm grinding bead was added to each tube. Then, 400 µL of extraction solution (methanol/water = 4:1, (*v*:*v*)) containing 0.02 mg/mL of internal standard (L-2-chlorophenylalanine) was used for metabolite extraction. Kohlrabi was ground by the frozen tissue grinder (Wonbio-96C, Shanghai Wanbo Biotechnology, Shanghai, China) for 6 min at −10 °C and 50 Hz, followed by low-temperature ultrasonic extraction (KW-100TDV, Kunshan Shumei, Kunshan, China) for 30 min at 5 °C and 40 kHz. The samples were stored at −20 °C for 30 min and centrifuged (H1850R, Cence, Changsha, China) at 13,000× *g* for 15 min at 4 °C. Finally, 100 µL of the supernatant was transferred to the injection vial for LC-MS/MS analysis.

2.3. Detection of Non-Volatile Metabolites by LC-MS/MS

The LC-MS/MS analysis for kohlrabi extract was performed on a Thermo UHPLC-Q Exactive HF-X system equipped with an ACQUITY HSS T3 column (100 mm × 2.1 mm i.d., 1.8 µm; Waters, Milford, MA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The mobile phases consisted of 0.1% formic acid in water/acetonitrile solution (95:5, *v*/*v*) (solvent A) and 0.1% formic acid in acetonitrile:isopropanol/water solution (47.5:47.5:5 , *v*/*v*/*v*) (solvent B). Positive ion mode separation gradient: 0–3 min, mobile phase B increased from 0% to 20%; 3–4.5 min, mobile phase B raised from 20% to 35%; 4.5–5 min, mobile phase B increased from 35% to 100%; 5–6.3 min, mobile phase B

maintained at 100%; 6.3–6.4 min, mobile phase B decreased from 100% to 0%; 6.4–8 min, mobile phase B maintained at 0%. Separation gradient in negative ion mode: 0–1.5 min, mobile phase B raised from 0% to 5%; 1.5–2 min, mobile phase B increased from 5% to 10%; 2–4.5 min, mobile phase B enhanced from 10% to 30%; 4.5–5 min, mobile phase B increased from 30% to 100%; 5–6.3 min, mobile phase B linearly maintained 100%; 6.3–6.4 min, the mobile phase B decreased from 100% to 0%; 6.4–8 min, the mobile phase B linearly maintained at 0%. The flow rate was 0.40 mL/min, and the column temperature was 40 °C.

MS conditions:

The mass spectrometric data were collected using a Thermo UHPLC-Q Exactive HF-X Mass Spectrometer equipped with an electrospray ionization (ESI) source operating in positive and negative modes. The optimal conditions were source temperature at 425 °C; sheath gas flow rate at 50 arb and aux gas flow rate at 13 arb; ion-spray voltage floating (ISVF) at −3500 V in negative mode and 3500 V in positive mode, respectively. Normalized collision energy, 20–40–60 V rolling for MS/MS. Full MS resolution was 60,000, and MS/MS resolution was 7500. Data acquisition was performed with the Data Dependent Acquisition (DDA) mode. The detection was carried out over a mass range of 70–1050 m/z.

2.4. Data Analysis

The pretreatment of LC/MS raw data was performed by Progenesis QI (Waters Corporation, Milford, MA, USA) software, and a three-dimensional data matrix in CSV format was exported. Internal standard peaks, as well as any known false positive peaks, were removed from the data matrix, deredundant and peak pooled. At the same time, the metabolites were identified by searching databases. The primary databases used were the HMDB (http://www.hmdb.ca/ (accessed on 9 January 2023)), Metlin (https://metlin.scripps.edu/ (accessed on 18 March 2023)), and Majorbio Database.

The R package "ropls" (Version 4.2.1, R Foundation for Statistical Computing, Vienna, Austria) was used to perform PCA and OPLS-DA and 7-cycle interactive validation evaluating the stability of the model. The non-volatile metabolites with VIP > 1, $p < 0.05$ were determined as significantly differential non-volatile metabolites based on the Variable Importance in the Projection (VIP) obtained by the OPLS-DA model and the p-value generated by Student's t test.

Differential non-volatile metabolites among the three groups were mapped into their biochemical pathways through metabolic enrichment and pathway analysis based on the KEGG database (http://www.genome.jp/kegg/ (accessed on 21 February 2023)). Python packages "scipy.stats" (https://docs.scipy.org/doc/scipy/ (accessed on 5 March 2023)) was used to perform enrichment analysis to obtain the most relevant biological pathways for experimental treatments.

3. Results and Discussion

3.1. PCA Analysis for Metabolomics of Kohlrabi

Based on LC-MS/MS combined with multivariate statistical methods, the differential non-volatile metabolites of the three kohlrabies were investigated. Quality control (QC) is usually required to obtain high-quality metabolomic data. In this work, 20 μL of each extract from 5Y, 8Y, and 11Y kohlrabi were mixed, and the obtained mixture was used as a QC. The aggregation degree of the QC was better than that of the 5Y, 8Y, and 11Y kohlrabi (Figure 1A), suggesting that the obtained experimental data were reliable. All data for the 5Y, 8Y, and 11Y kohlrabi were well distributed in different regions, indicating significant differences in the non-volatile metabolites among the kohlrabies. R^2_X and Q^2 were the main parameters for judging the PCA model. In this study, $R^2_X = 0.628 > 0.5$ and $Q^2 = 0.367$, indicating that the PCA model could well interpret the significant differences among the differential non-volatile metabolites in the kohlrabies. Figure 1B showed the distribution and contribution of metabolites on different principal components.

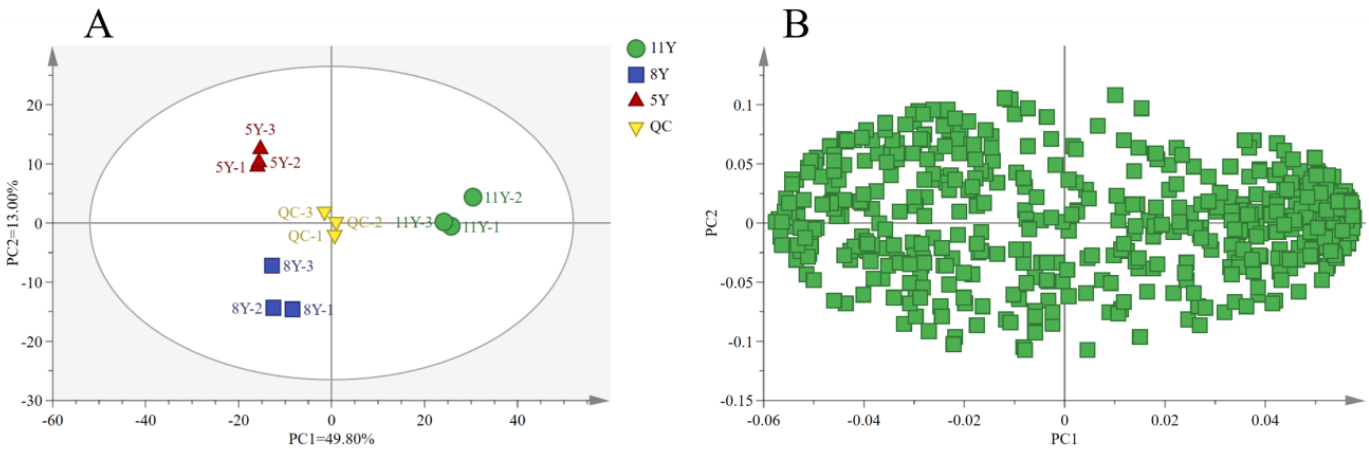

Figure 1. The scatter plots (**A**) and loading plots (**B**) of the PCA model for the 5Y, 8Y, and 11Y fermented kohlrabi. 5Y: 5-year, 8Y: 8-year, 11Y: 11-year.

3.2. OPLS-DA Analysis of Non-Volatile Metabolites from Kohlrabi by LC-MS/MS

For further precise analysis of the three groups of kohlrabi (A1, A2, and A3), OPLS-DA analysis was consequently employed, as shown in Figure 2. Figure 2A,C,E implied the OPLS-DA score maps for the three groups of kohlrabi, respectively. It can be seen clearly that all groups were significantly separated, suggesting that the significant differences in non-volatile metabolites were caused by the different fermentation periods. Moreover, it has been reported that changes in metabolites in fermented kohlrabi might be due to the long-term strong activity of salt-tolerant microorganisms [17]. The OPLS-DA model parameters are listed in Table 1. The R^2_X and R^2_Y values for A1, A2, and A3 were over 0.5. The Q^2 values for the three groups of kohlrabi were nearly 1.0, which could explain and predict the differences between every two kohlrabies' non-volatile metabolites.

In order to further demonstrate that the model was reliable, 200-loop-iteration permutation tests were consequently conducted (Figure 2B,D,F). All the intersections for the Q^2 regression line and Y-axis were on the negative half-axis, suggesting that the OPLS-DA model was stable and reliable. On the other hand, no overfitting phenomenon was observed.

Table 1. Parameters of the OPLS-DA models for the three different groups of kohlrabi.

Group	R^2_X (cum)	R^2_Y (cum)	Q^2 (cum)
A1	0.728	1.000	0.984
A2	0.751	1.000	0.986
A3	0.678	1.000	0.969

3.3. Screening of Differential Non-Volatile Metabolites in Kohlrabi

A total of 601 non-volatile metabolites were detected (Table S1), and 444 non-volatile metabolites were identified in the three groups, as shown in Figure 3. Organic acids and derivatives, organoheterocyclic compounds, benzenoids, lipids and lipid-like molecules, and organic oxygen compounds were the main metabolites, accounting for 27.93%, 16.22%, 15.54%, 14.64%, and 14.19%, respectively. Organic acids and derivatives accounted for the highest proportion, and a total of 124 metabolites were matched.

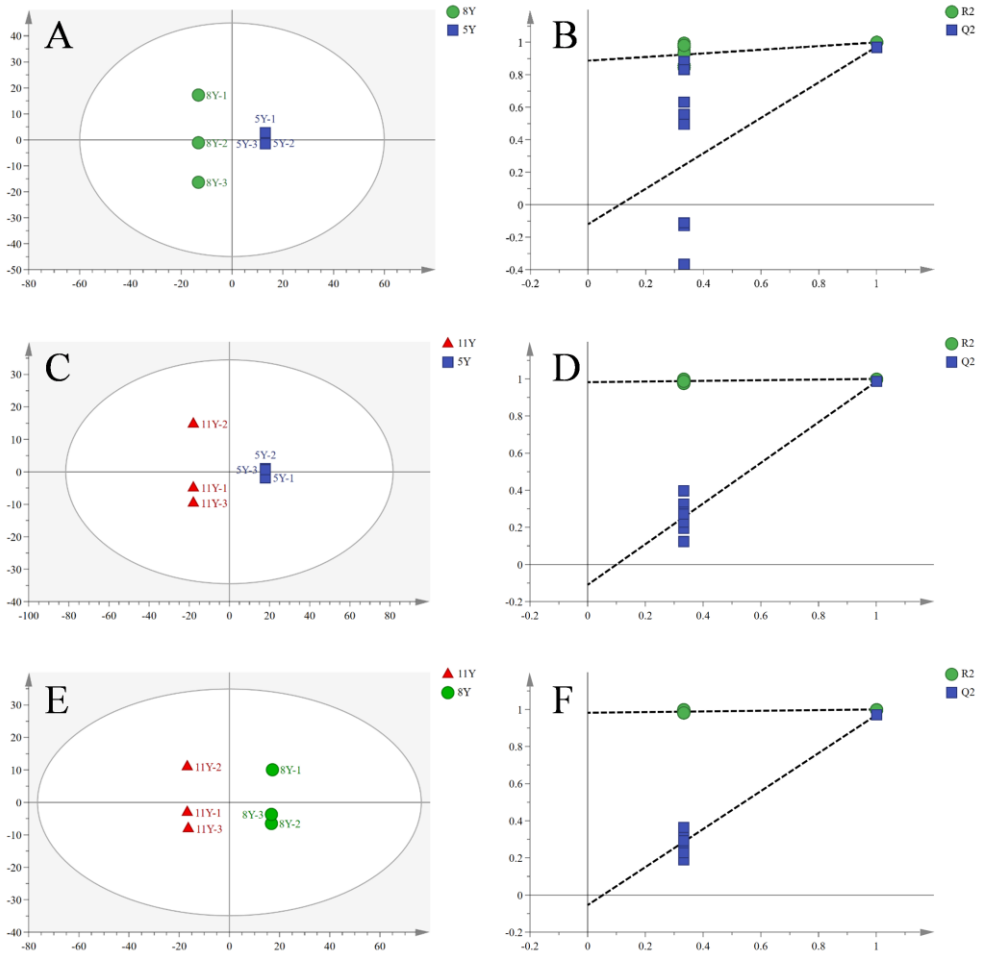

Figure 2. Scatter plots and permutation tests of the OPLS-DA models for the three groups of kohlrabi. (**A**,**C**,**E**) represent the OPLS-DA analyses for A1 (5Y–8Y), A2 (5Y–11Y), and A3 (8Y–11Y), while (**B**,**D**,**F**) represent permutation tests for A1 (5Y–8Y), A2 (5Y–11Y), and A3 (8Y–11Y).

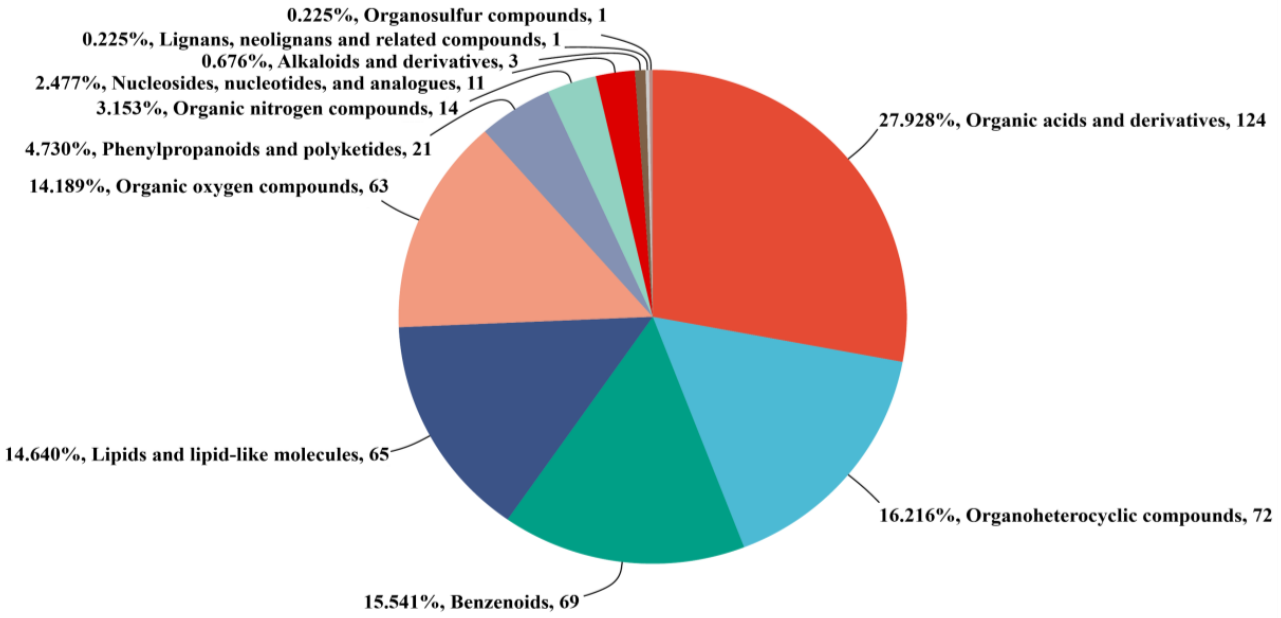

Figure 3. Classification of non-volatile metabolites in the three groups of kohlrabi.

In this study, the non-volatile metabolites with VIP > 1, $p < 0.05$, and $|\log_2 FC| > 1$ were defined as significantly differential metabolites. According to the set standards, 31, 169, and 123 differential non-volatile metabolites were, respectively, screened from the three groups, as shown in Figure 4A. With the extension of fermentation, microorganisms metabolized proteins, polysaccharides, fat and other nutrients in kohlrabi to produce organic acids, amino acids, fatty acids, and other products through enzyme catalysis [18]. A2 (5Y–11Y) had a longer fermentation interval than A1 (5Y-8Y) and A3 (8Y-11Y); the types and quantities of differential metabolites were consequently more abundant than those in A1 and A3. The differential non-volatile metabolites of the three groups were analyzed using a Venn diagram, as seen in Figure 4B. Eleven differential metabolites were screened from the three groups, including four organic acids and derivatives (ectoine, aminomalonic acid, diaminopimelic acid, and L-phenylalanine), two lipids and lipid-like molecules (androsterone and 9,10,13-TriHOME), two organoheterocyclic compounds (biotin and 6-hydroxymelatonin), two benzenoids (labetalol and 4-nitrocatechol), and one organic oxygen compounds (suprofen). On the other hand, these differential metabolites increased with the extension of the fermentation period, which was consistent with Liu's study [19].

The composition and content of organic acids are important factors affecting the flavor of fermented foods. Lactic acid, acetic acid, malic acid, citric acid, tartaric acid, fumaric acid, and other organic acids were detected in the fermented kohlrabi. Lactic acid and acetic acid are the most important organic acids, usually produced by *Lactobacilli*, and participate in a variety of biochemical reactions. It has been well documented that lactic acid is the main source of flavor, which is crucial for the formation of the sour taste in fermented foods [20]. In this work, the lactic acid content in 8Y kohlrabi was significantly higher than those in 5Y and 11Y kohlrabi ($p < 0.05$), suggesting that the lactic acid content probably increased in the earlier stage and decreased in the later stage. It might be due to the large amount of lactic acid accumulated by lactic acid bacteria metabolizing carbohydrates in the earlier stage. However, in the later stage of fermentation, due to the reduction of nutrients, the lactic acid was metabolized as a carbon source by microorganisms, decreasing the lactic acid [21]. The higher amount of lactic acid in 8Y kohlrabi made it more suitable for making appetizing acid soup. It is well known that acetic acid mainly comes from *Bifidobacterium* fermentation. The increase of acetic acid enhanced the sour taste of kohlrabi and gave the flavor characteristics related to vinegar, spicy and sour taste at high concentrations [22]. The acetic acid content in the three fermented kohlrabies was higher than that of lactic acid, probably due to the difference in lactic acid bacteria in the fermentation system [23]. In addition to giving a unique sour taste to fermented foods, organic acids can also serve as substrates to form various flavor substances such as ketones, alcohols, aldehydes, and esters.

On the other hand, organic acids have a significant impact on plant life metabolism. It has been suggested that citric acid can act as a chelating agent to enhance the absorption of iron and manganese by reeds [24]. In contrast, malic acid was considered to increase the tolerance of plants to water stress [25]. In this study, a lot of free acid acids were also detected, among which L-leucine, L-methionine, L-phenylalanine, L-proline, L-aspartic acid, L-tryptophan, L-tyrosine, L-valine, and L-glutamic acid increased continuously with the extension of fermentation. The fresh taste is a pleasant salty taste, and L-aspartic acid and L-glutamic acid are considered the main sources of fresh taste [26]. The 11Y kohlrabi had the highest L-aspartic acid and L-glutamic acid content, possibly explaining why the 11Y kohlrabi was more suitable for cooking fresh soup.

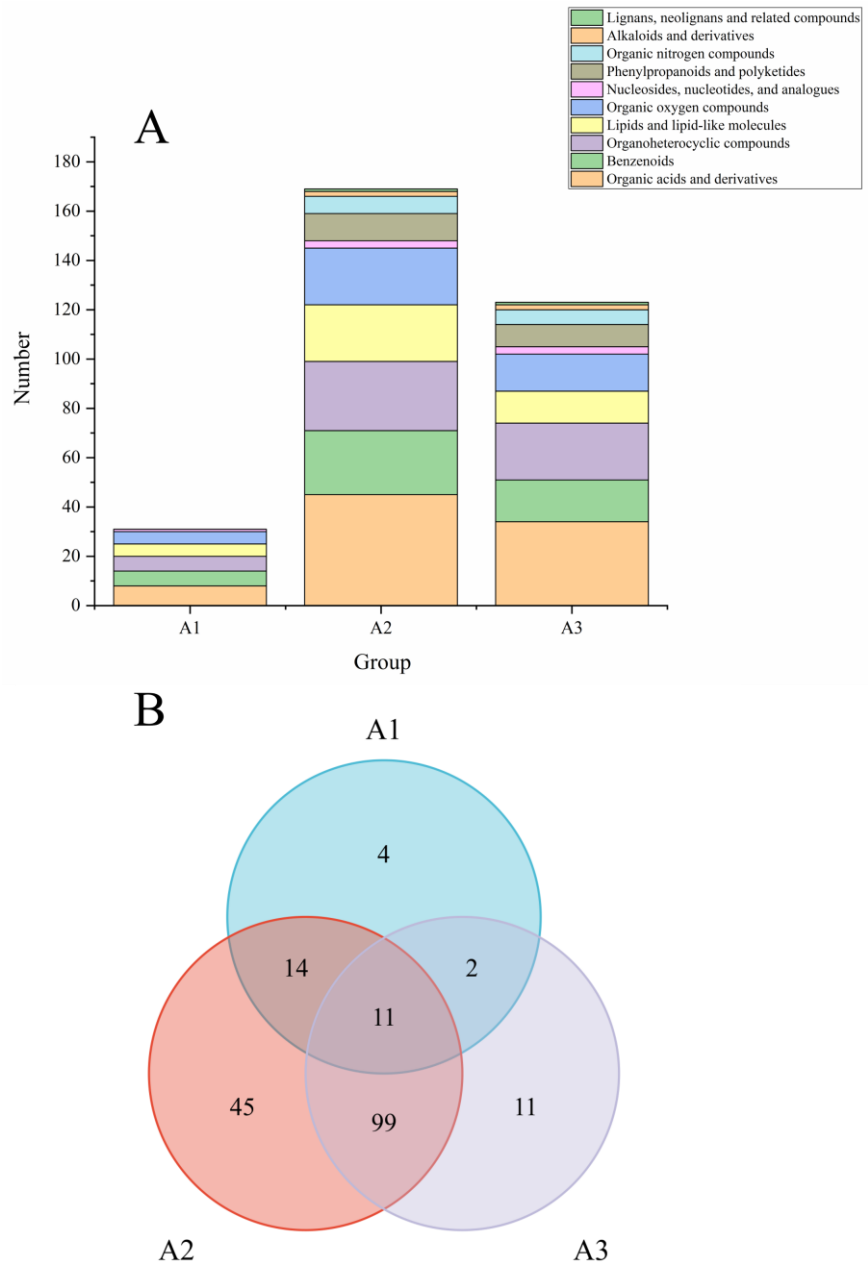

Figure 4. Classification information (**A**) and Venn diagram (**B**) for the differential non-volatile metabolites in the three groups of kohlrabi (A1: 5Y and 8Y, A2: 5Y and 11Y, A3: 8Y and 11Y).

Organoheterocyclic and organic oxygen compounds mainly come from microbial metabolism, including carbohydrates, nucleic acids, alkaloids, pigments, vitamins, and other secondary metabolites. Carbohydrates are the basis for the sweetness of fermented kohlrabi. In this study, glucose, sucrose, fructose, lactose, and other substances were detected in the fermented kohlrabi. Glucose and fructose are commonly considered the main available energy and carbon sources for *Lactobacillus*, most of which were utilized during the fermentation. However, the soluble reducing sugar such as glucose, fructose, galactose, and xylose gradually increased during the fermentation of kohlrabi, which was inconsistent with Hashemi's experimental results [27]. This was probably due to the microorganisms related to starch metabolism, and amylase maintained high activity during fermentation, and thus the starch in kohlrabi was constantly used to produce carbohydrates [28]. Xylose is an excellent catalyst for inducing the Maillard reaction, which can significantly improve the fresh taste and meat flavor of foods [29]. Alkaloids are heterogeneous families of plant secondary

metabolites, mainly including isoquinoline, pyridines, and others, with nitrogen-containing heterocycles in their basic structures. In this study, three alkaloid differential metabolites, termed harmala, ecgonine methyl ester, and morphinone, were detected. Secondary metabolites are synthesized into organic compounds with a large number of primary metabolites as precursors through sugar metabolism, Shikimic acid extension, acetic acid derivation, and amino acid derivation [30]. Biotin is an important auxiliary factor for many carboxylation and decarboxylation reactions, which increased significantly during the fermentation of kohlrabi. In addition, biotin plays an essential role as a carboxyl carrier in the carboxylation reaction of metabolic pathways such as gluconeogenesis, fatty acid synthesis, and amino acid decomposition [31].

Benzenoids were the primary differential metabolites detected in this study, most of which were phenolic compounds. Phenols are important secondary metabolites and natural antioxidant components in plants, exhibiting strong antioxidant activity in the metabolic process [32]. During the fermentation, phenolic compounds significantly increased, possibly due to the high permeability environment caused by high salt content and the release of phenolic substances from the kohlrabi substrate [33]. Moreover, microorganisms in the fermentation system could convert bound phenols and macromolecular phenols into free and small molecule phenols through metabolic pathways [34].

Lipids are the main components of eukaryotic cell membranes in plants, which are crucial for the life activities of plants [35]. The main lipid metabolites detected in fermented kohlrabies were fatty acyls, steroids, and steroid derivatives. Most of the fatty acyls increased during the fermentation, with fatty acids and aggregates accounting for a higher proportion. Among the detected differential non-volatile metabolites, only 13-L-hydroperoxylinoleic acid and 9(S)-HPODE were lineolic acids and derivatives. They significantly increased with the extension of the fermentation period, with the highest content in the 11Y kohlrabi. The 13-L-hydroperoxylinoleic acid and 9(S)-HPODE in 11Y kohlrabi were 2.82 and 8.09 times higher than those in 5Y kohlrabi, respectively. Lipids are used as precursors for many compounds during fermentation, which can be metabolized to substances such as lactic acid, acetic acid, and acetone by microorganisms [36]. *Lactobacilli*, *Staphylococcus*, *Enterococcus*, and *Pediococcus* are demonstrated to correlate with lipid metabolism [37]. It has been reported that *lactobacilli* can produce fatty acid hydratases, participating in synthesizing fatty acid derivatives [38].

There were significant differences in metabolite content among the three different groups of kohlrabi, leading to different diets. Free amino acids are the main contributors to flavor formation, mainly derived from protein degradation. The free amino acids, including L-leucine, L-methionine, L-phenylalanine, L-proline, L-threonine, L-tryptophan, L-tyrosine, L-valine, and leucine, increased with the extension of fermentation. Furthermore, the 11Y kohlrabi had the highest lineolic acids and derivatives, and soluble sugars, such as sucrose, glucose, fructose, and galactose, compared to 5Y and 8Y kohlrabies. 11Y kohlrabi was usually used for cooking fresh soups, possibly due to the abundant L-aspartic acid and L-glutamic acid, as well as soluble sugars, which were beneficial for improving the freshness and flavor of soups. 8Y kohlrabi produced more organic acids than that of 5Y and 11Y kohlrabi, with the highest lactic acid content among the three groups of kohlrabi. Lactic acid provides a sour taste and can interact with alcohols, aldehydes, and ketones to produce various new flavor substances during the fermentation of vegetables [39]. Consequently, 8Y kohlrabi was suitable for making sour soups. 5Y kohlrabi had more proteins and carbohydrates, which would be utilized through the Maillard reaction to form a large number of products, contributing to forming unique volatile flavor substances of kohlrabi [29] since higher moisture content will dilute the concentration of Maillard reaction substrate, which thus will inhibit the reaction process [40,41], making 5Y kohlrabi suitable for cooking braised dishes.

3.4. The Common Differential Metabolites in the Three Groups of Kohlrabi

Eleven common differential metabolites in the three groups of kohlrabi were screened (VIP > 1, $p < 0.05$, and $|\log_2 FC| > 1$), as shown in Figure 5. The contents of the 11 metabo-

lites in the three groups of kohlrabi varied significantly, which could be used as potential biomarkers to distinguish the 5Y, 8Y, and 11Y kohlrabi. 5Y kohlrabi had the highest levels of diaminopimelic acid, ectoine, and labetalol. Diaminopimelic acid and ectoine decreased significantly with the extension of fermentation. Diaminopimelic acid might be used to synthesize lysine, leading to its decrease [42]. Ectoine is generally produced by some halophilic and salt-tolerant microorganisms and serves as an efficient solute with osmotic regulation. In addition, it is also used as an effective stabilizer for nucleic acids, DNA protein complexes, and enzymes [43]. The differential metabolite with higher content in the 8Y group was 9,12,13-TriHOME, which increased in the earlier stage and decreased in the later stage. The main product of linoleic acid oxidation is trihydroxyoctadecenoic acid (TriHOMEs), which is the isomer of 9,10,13-TriHOME [44]. Linoleic acid of kohlrabi was probably oxidized and decomposed into TriHOMEs with a bitter taste during fermentation. However, the TriHOMEs decreased with the extension of fermentation. It was possibly due to the lack of enzymes required for oxidizing and decomposing linoleic acid to generate TriHOMEs in the later stage of fermentation, agreeing with a previous study [45]. 11Y kohlrabi had more 4-nitrocatechol, biotin, aminomalonic acid, 6-hydroxymelatonin, androsterone, suprofen, and L-phenylalanine, which increased gradually during the fermentation.

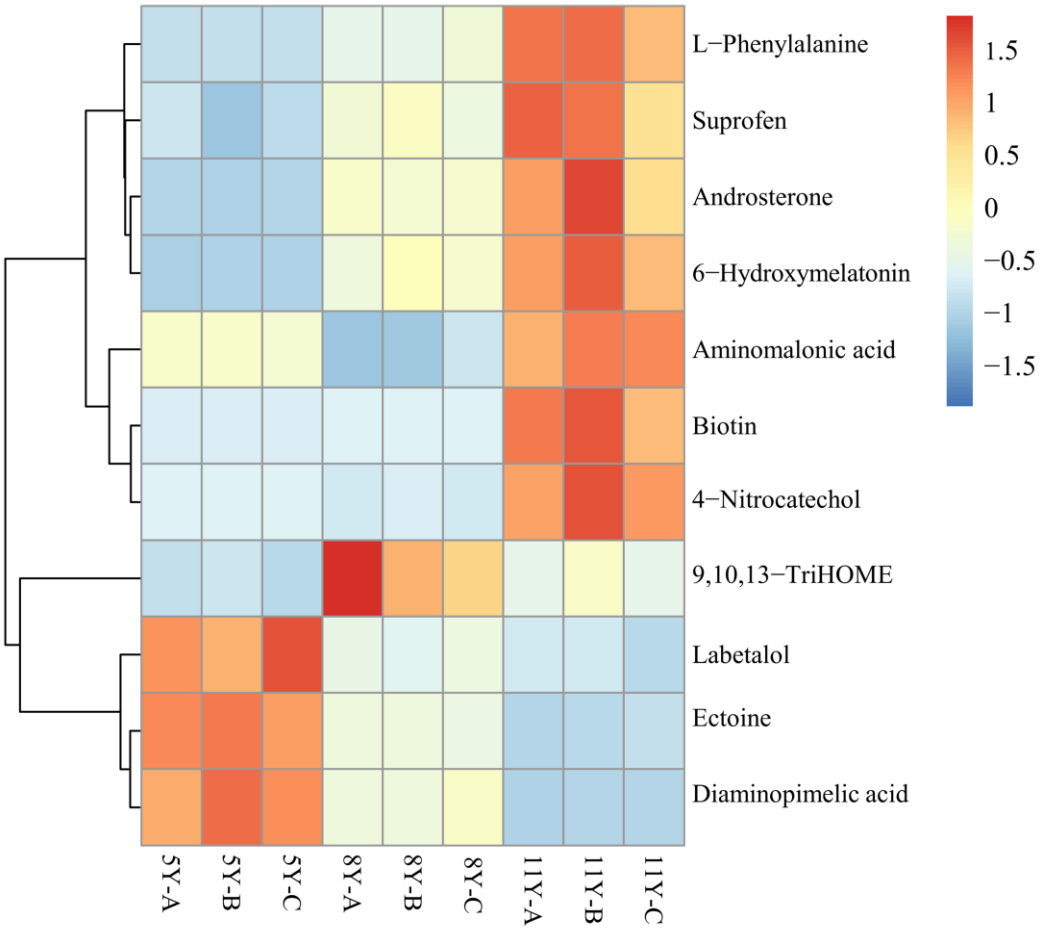

Figure 5. Hierarchical clustering analysis heatmap of main differential metabolites in fermented kohlrabies.

3.5. Metabolic Pathway Analysis for Differential Metabolites in Kohlrabi

Based on the selected 11 differential metabolites, a general overview of the pathways of the differential metabolite enrichment is shown in Figure 6. Six pathways were enriched with p-value < 0.01 and impact value >0.1, termed aminoacyl-tRNA biosynthesis (Impact = 0.11), pantothenate and CoA biosynthesis (Impact = 0.14), citrate cycle (Impact = 0.24), glyoxylate

and dicarboxylate metabolism (Impact = 0.21), glycine, serine and threonine metabolism (Impact = 0.18), and lysine biosynthesis (Impact = 0.36).

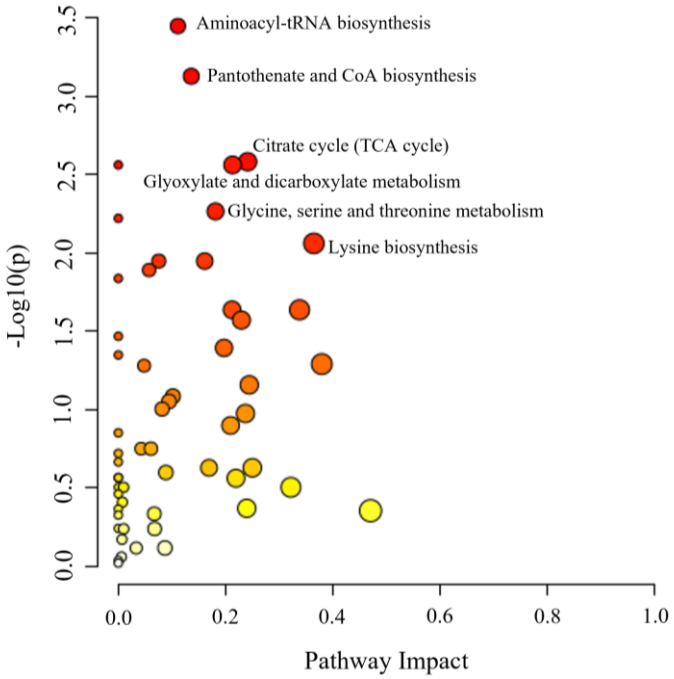

Figure 6. Enrichment analysis of metabolic pathways of differential substances in fermented kohlrabies. A larger circle means a larger impact factor, and vice versa, a smaller impact factor. The redder the color of the circle, the smaller the *p*-value.

Four metabolic pathways were integrated (Figure 7 and Table S2): citrate cycle, glycine, serine and threonine metabolism, pantothenate, CoA biosynthesis, glycoxylate, and dicarboxylate metabolism. Citrate cycle metabolized pyruvate produced by the glycolysis pathway as the substrate to generate acetyl-CoA under the catalysis of pyruvic acid decarboxylase complex [46]. L-malate was converted into oxaloacetate catalyzed by malate dehydrogenase, which reacted with acetyl CoA to produce citrate under the induction of citrate synthase. Citrate subsequently entered the glyoxylate cycle to generate isocitrate, which was then converted into oxaloacetate under the catalysis of isocitric acid dehydrogenase. The α-ketoglutaric acid was finally produced by β-decarboxylation. The glycine, serine, and threonine metabolism pathway used L-serine as substrate. Some of the L-serine was converted into pyruvate catalyzed by serine dehydratase and subsequently entered the citrate cycle. While part of the L-serine was combined with indole, converted into L-tryptophan, and then entered tryptophane metabolism. The rest of the L-serine exchanged acyl groups with phosphatidyl ethanolamine to generate phosphatidylserine and then entered the glycerophospholipid metabolism pathway to produce choline. Choline was oxidized to betaine aldehyde under the catalysis of choline monooxygenase, which was finally converted into betaine by the catalysis of betaine dehydrogenase. The pantothenate and CoA biosynthesis pathways metabolized pyruvate as substrate, which was converted into (S)-2-acetolactate catalyzed by acetate synthesis. The (S)-2-acetolactate was reduced to (R)-2,3-dihydroxyisovaleric acid by ketol-acid reductoisomerase, which subsequently converted into α-ketovaline by dihydroxy acid dehydratase. Finally, the L-valine was generated by branched-chain amino acid aminotransferase from α-ketovaline. L-malate, oxalacetate, citrate, *cis*-aconate, isocitate, and glyoxylate in the glyoxylate and dicarboxylate metabolism pathways formed the glyoxylate cycle, which interconnected with the citrate cycle pathway through glyoxylate.

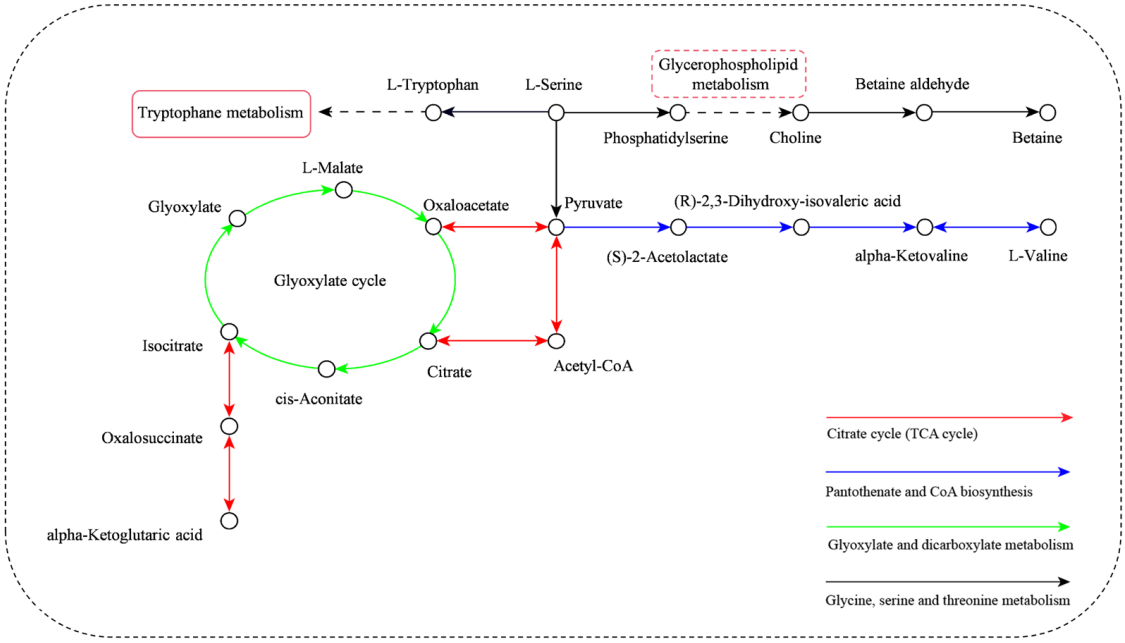

Figure 7. Integrated analysis diagram of main metabolic pathways.

In this study, the content of pyruvate showed a trend of increasing first and then decreasing. It might be because, in the earlier stage, microorganisms decomposed sugars in kohlrabi through glycolysis and other metabolic pathways to generate pyruvic acid. However, in the later fermentation stage, organic acids greatly accumulated due to the growth of microorganisms, leading to decreased pH. As a weak acid, pyruvic acid was prone to protonation at a low pH value; thus, its content was decreased [47]. The pyruvic acid was decomposed and utilized to produce new products, such as various amino acids. This could be confirmed by the significant increase in the content of various metabolites and L-valine on the pantothenate and CoA biosynthesis pathways. Therefore, the content of pyruvic acid was consequently relatively reduced in the later stage of fermentation.

Environmental stress, especially salt stress, adversely affects plant carbohydrate metabolism, and the accumulation of sugars and alcohols plays a vital role in osmotic regulation and carbon storage [48]. It has been reported that citrate cycle activity will undergo significant changes to adapt to the environment under various stress conditions [49]. α-ketoglutamic acid is a key compound in the nitrogen and carbon metabolic pathways, which connects the citrate cycle with other metabolic pathways, such as amino acid, gibberellin, glucosinolate, and alkaloid biosynthesis [50]. Abiotic stress, such as salinity and dryness, can induce the accumulation of proline, glycine, betaine, and sugar, which act as osmotic protective agents in different halophytes [51]. Glycine, serine, and threonine metabolic pathways are probably related to salt stress [52]. The flavor of fermented kohlrabi mainly comes from the metabolism of microorganisms, including organic acids, aldehydes, and esters. Under a high salt environment, the metabolism of many microorganisms is inhibited. However, betaine can significantly promote the maintenance of osmotic balance to remove this inhibition, which makes the microorganisms can carry out normal metabolism to synthesize rich flavor substances in the fermentation of kohlrabi [53]. Betaine is a secondary metabolite produced by choline oxidation on the glycine, serine and threonine metabolism pathway and widely exists in animals and plants [54].

4. Conclusions

In this study, LC-MS/MS coupled with multivariate statistical analysis was employed to explore the non-volatile differential metabolites of 5Y, 8Y, and 11Y Chinese kohlrabi. With the standard of VIP > 1, $p < 0.05$, $|\log_2 FC| > 1$, 31, 169 and 123 differential metabolites

were identified in A1 (5Y–8Y), A2 (5Y–11Y), and A3 (8Y–11Y). The differential metabolites were organic acids and derivatives, organoheterocyclic compounds, benzenoids, lipids and lipid-like molecules, and organic oxygen compounds. Eleven common metabolites were screened from the Veen diagram, and four key metabolic pathways significantly correlated with the differential metabolites were then enriched.

Complex biochemical reactions took place during the ultra-long-term fermentation of kohlrabi. A large amount of free amino acids and soluble sugars made 11Y kohlrabi suitable for making fresh soup. There were more organic acids and the highest content of lactic acid in 8Y kohlrabi, making it suitable for cooking appetizing sour soups. The 5Y kohlrabi was rich in protein and sugar substances, which could form unique flavors through the Maillard reaction, making it suitable for the processing of braised dishes. Deeply investigating the metabolites and their changes during ultra-long-term fermentation is the next step of work, which is important for the development of kohlrabi-containing dishes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9080753/s1, Table S1: Metabolite peak ratio of kohlrabi in 5Y, 8Y and 11Y groups; Table S2: Metabolites enriched by four metabolic pathways.

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