

Determining the changes in metabolites of *Dendrobium officinale* juice fermented with starter cultures containing *Saccharomycopsis fibuligera* FBKL2.8DCJS1 and *Lactobacillus paracasei* FBKL1.3028 through untargeted metabolomics

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Abstract

Background

The present study aimed to investigate the changes in volatile components and metabolites of *Dendrobium officinale* (*D. officinale*) juice fermented with starter cultures containing *Saccharomycopsis fibuligera* and *Lactobacillus paracasei* at 28 °C for 15 days and post-ripened at 4 °C for 30 days using untargeted metabolomics of liquid chromatography-mass spectrometry (LC-MS) and headspace solid-phase microextraction-gas chromatography (HS-SPME-GC-MS) before and after fermentation.

Results

The results showed that the alcohol contents in the *S. fibuligera* group before fermentation and after fermentation were 444.806 ± 10.310 µg/mL and 510.999 ± 38.431 µg/mL, respectively. While the alcohol content in the fermentation broth group inoculated with the co-culture of *L. paracasei*+ *S. fibuligera* was 504.758 ± 77.914 µg/mL, with a significant increase in the contents of 3-Methyl-1-butanol, Linalool, Phenylethyl alcohol, and 2-Methyl-1-propanol. Moreover, the Ethyl L (-)-lactate content in the co-culture *L. paracasei*+ *S. fibuligera* group (7.718 ± 6.668 µg/mL) was higher than those in the *L. paracasei* (2.798 ± 0.443 µg/mL) and *S. fibuligera* monoculture groups (0 µg/mL). The co-culture of *L. paracasei*+ *S. fibuligera* significantly contributed to the metabolic production of ethyl L (-)-lactate in *D. officinale* juice. The total number of differential metabolites screened after fermentation mainly included alcohols, organic acids, amino acids, nucleic acids, and their derivatives. Of them, twenty-three metabolites were significantly up-regulated in the ten key metabolic pathways of the co-culture *L. paracasei*+ *S. fibuligera* group, including 11 types of acids. Furthermore, the metabolic pathways, such as pentose and glucuronate interconversions, the biosynthesis of alkaloids derived from terpenoid and polyketide, and aminobenzoate degradation were significantly upregulated in this group. These three metabolic pathways facilitate the synthesis of bioactive substances, such as terpenoids, polyketides, and phenols, and enrich the flavor composition of *D. officinale* juice.

Conclusions

The study results demonstrated that the co-culture of *L. paracasei*+ *S. fibuligera* could promote the flavor harmonization of fermented products, and provide a theoretical basis for the study of the flavor of *D. officinale* juices and the functional investigation of fermentation metabolites.

Background

Fermentation is a metabolic process characterized by the release of different chemicals named metabolites through the action of microorganisms [1]. *Lactobacillus* and yeast, widely acknowledged as probiotics, are the most common microorganisms in food fermentation. For instance, *Lactobacillus*

Rhamnosus, *Lactobacillus Plantarum*, and yeast co-cultures could inhibit the growth of pathogenic bacteria, neutralize enterotoxins, and develop antibiotics resistance [2, 3]. It is reported that the co-culture of lactic acid bacteria and yeasts could enhance sugar metabolism and improve aroma formation during the fermentation of cocoa beans [4]. During fermentation, yeast provides the vitamin, amino acids, and other necessary growth factors for lactic acid bacteria, and the end products of lactic acid bacteria metabolism are utilized as an energy source by the yeasts [5]. The synergistic effect of these microorganisms in the fermentation process can improve the palatability, sensory properties, and utilization rate of nutrients in food and extend the shelf life of food.

Since ancient times, Chinese medicine has been based on the theory that "medicine and food have the same origin," which means many foods can act as medicines with equivalent effectiveness in preventing and treating diseases [6]. Many medicinal plants have been used in the health food industry, such as *Radix Codonopsis*, *D. officinale*, *Astragalus*, *Ganoderma*, *Amanita*, *Cornu Cerevisiae*, *Eucommiae*, etc. Of them, *D. officinale* is one of the prominent medicinal food plants, containing various bioactive ingredients, such as polysaccharides, bibenzoids, phenolics, alkaloids, flavonoids, etc. Research shows that *D. officinale* possesses anti-tumor, gastrointestinal protection, hypoglycemic, anti-aging, and anti-osteoporosis effects [7, 8]. Tian et al. fermented *D. officinale* with *Bacillus* sp. DU-106 and found that DU-106 could change the monosaccharide composition and molecular weight of *D. officinale* polysaccharides and promote immune stimulating activity, elucidating the benefits of treating *D. officinale* by fermentation [9]. However, the current research on *D. officinale* is mainly focused on the extraction and application of natural bioactive components, while there are fewer reports on the fermentation of *D. officinale* using probiotics [10]. Therefore, in this study, *D. officinale*, as a raw material, was inoculated with lactic acid bacteria and yeast for liquid fermentation. The changes and differences in compounds before and after fermentation of *D. officinale* juice were investigated by headspace solid-phase microextraction-gas chromatography-mass spectrometry and non-targeted metabolomics liquid chromatography-mass spectrometry, combined with multivariate statistical analyses to screen the differential metabolites and analyze the relevant metabolic pathways, active components, and aroma components of *D. officinale* juice. The study results will provide an effective research strategy for analyzing the functional and aroma characteristics of *D. officinale* juice.

Materials And Methods

Materials and reagents

Dendrobium officinale were obtained from Guizhou Qiansheng Taijiang Poverty Alleviation Industry Development Co., LTD (Guizhou, China); *Saccharomycopsis fibuligera* FBKL2.8DCJS1, isolated and screened from Wild Grape (Guizhou, China), conserved in China Center for Type Culture Collection (Wuhan, China), No. CCTCC NO: M2020891; *Lactobacillus paracasei* FBKL1.3028, isolated and screened from White Sour Soup (Guizhou Province, China), conserved in China Center for Type Culture Collection (Wuhan, China), No. CCTCC NO: M2020156; MRS broth medium, MRS agar medium, YEPD medium, and YPD medium were obtained from Shanghai Bo Microbiology Technology Co., Ltd (Shanghai, China);

Liquid malt (100% malt) was obtained from Gansu Hongli Biotechnology Co., Ltd,(Gansu, China); Glucose was obtained from Hebei Jinfeng Starch Glycol Co., Ltd (Hebei, China); α - High temperature amylase and saccharifying enzyme were obtained from Solarbio (Beijing, China); Pectinase was obtained from Manson Trading Co., Ltd (France); 2-octanol, German Dr Ehrenstorfer GmbH (Germany); Acetonitrile Thermo; Formic acid was obtained from TCI; Ammonium formate was obtained from Sigma-Aldrich (Shanghai, China).

Fermented *D. officinale* juice

S. fibuligera in glycerol tubes at -80°C was activated in YPD medium at 28°C for 48 h, and *L. paracasei* was activated in MRS solid medium at 37°C for 48 h. The activated strains were inoculated into sterilized and cooled liquid malt extract solution (128 g of liquid malt extract, 875 mL of distilled water in four 500 mL conical flasks, fixed at 115°C for 20 min). *S. fibuligera* was fixed at 28 °C for 36 h at 160 r/min and *L. paracasei* was fixed at 37 °C for 36 h at 160 r/min. *D. officinale* fresh strips were dried and crushed through a 40-mesh sieve. Then, 60 g of *D. officinale* powder was added to 4 L of distilled water and boiled for one h. Accurately, 4 g of α -high temperature amylase was added to the pot and enzymatically digested at 95 °C for 1 h. Accurately, 2 g of pectinase and 2 g of glycosylase were added to the pool and enzymatically digested at 55 °C for 1 h. 300 g of glucose was added, dissolved, filtered, encapsulated in 12 bottles, and sterilized at 115 °C for 20 min, which was regarded as the unfermented *D. officinale* juice. After cooling, 6% *L. paracasei*, 6% *S. fibuligera*, 4% *L. paracasei*, and 2% *S. fibuligera* were inoculated, respectively and three parallel samples were prepared for each piece (Table 1). The unfermented *D. officinale* juice was used as a blank control. After inoculation, the juice was incubated at 37 °C for 24 h, fermented at 28 °C for 15 days, and finally, post-ripened at 4 °C for 30 days to obtain the sample.

Table 1
The Addition amount of fermentation strain

Inoculation volume (%)	A	B	C	D
<i>L. paracasei</i>	0	0	6	4
<i>S. fibuligera</i>	0	6	0	2

Hs-spme-gc-ms Analysis

Each sample of the fermented and unfermented *D. officinale* juice was treated in triplicate. The volatile components of the *D. officinale* juice were determined by headspace solid-phase microextraction and gas chromatography. The volatile substances were detected by 7890A/5975C gas chromatography-mass spectrometer equipped with PAL automatic sampler and DB-WAX (30 m×0.25 mm; i.d.,0.25 μ m) capillary chromatographic column. Accurately 8 mL of the sample was added to a 20 mL sample bottle, along with 2 g of NaCl and 1 μ L of internal standard (2-octanol, 0.822 mg/mL by mass), and then the bottle was quickly sealed by tightening the cap. Later, the sample was equilibrated at 45°C for 10 min at 160 r/min. After the gas-liquid aromatic substances in the bottle reached the equilibrium state, the extraction

head was inserted into the headspace of the sample bottle. The extraction was stirred for 45 min to achieve gas-solid and gas-liquid balance of the aromatic substances in the sample bottle. The extraction head was then inserted into the GC-MS injection port and thermally desorbed at 250°C for 10 min, and the sample was injected in non-split mode. The column flow rate was 0.8 mL/min in a constant flow mode. The programmed temperature was increased from 40°C to 120°C at 10°C/min and held for 3 min, then to 230°C at 20°C/min for 67 min, the ion source temperature was 230°C, the ionization mode was electron ionization (EI), and the ion energy source was 70 eV with a mass scan range of 20 to 350 u [11].

Lc-ms Analysis

Liquid chromatography conditions: The LC analysis was performed on a Vanquish UHPLC System (Thermo Fisher Scientific, USA). Chromatography was carried out with an ACQUITY UPLC® HSS T3 (150 × 2.1 mm, 1.8 µm) (Waters, Milford, MA, USA), and the column was maintained at 40 °C. The flow rate and injection volume were set at 0.25 mL/min and two µL, respectively. As for the LC-ESI (+)-MS analysis, the mobile phases consisted (B2) 0.1% formic acid in acetonitrile (v/v) and (A2) 0.1% formic acid in water (v/v). Separation was conducted under the following gradients: 0 ~ 1 min, 2% B2; 1 ~ 9 min, 2%~50% B2; 9 ~ 12 min, 50%~98% B2; 12 ~ 13.5 min, 98% B2; 13.5 ~ 14 min, 98%~2% B2; 14 ~ 20 min, 2% B2. As for the LC-ESI (-)-MS analysis, The mobile phase consisted acetonitrile (B3) and 5mM of ammonium formate(A3). Separation was conducted under the following gradients: 0 ~ 1 min, 2% B3; 1 ~ 9 min, 2%~50% B3; 9 ~ 12 min, 50%~98% B3; 12 ~ 13.5 min, 98% B3; 13.5 ~ 14 min, 98%~2% B3; 14 ~ 17 min, 2% B3[12].

Mass spectrum conditions: Mass spectrometric detection of metabolites was performed on Q Exactive Focus (Thermo Fisher Scientific, USA) with an ESI ion source and simultaneous MS1 and MS/MS (Full MS-ddMS2 mode, data-dependent MS/MS) acquisition. The parameters were as follows: sheath gas pressure, 30 arb; aux gas flow, ten arb; spray voltage, 3.50 kV and - 2.50 kV for ESI(+) and ESI(-), respectively; capillary temperature, 325 °C; MS1 range, m/z 100–1000; MS1 resolving power, 70000 FWHM; the number of data dependent scans per cycle, 3; MS/MS resolving power, 17500 FWHM; normalized collision energy, 30 eV; dynamic exclusion time, automatic [13].

Volatile Component Data Analysis

All tests were performed in triplicate. The experimental data were collated and graphed using Excel 2010. IBM SPSS Statistics, 26 and Origin 2021 were used for graphical plotting. The significance analysis was performed using the Duncan test. $P < 0.05$ was considered statistically significant. TBtools was used for plotting the heat maps.

Differential Metabolite Analysis

The principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed using the R package Ropls [14] to reduce the dimensionality of the sample data, and plot the score, loadings, and S-plot plots to demonstrate the differences in metabolite composition among the samples. The model was tested for overfitting using a permutation test. The functional pathways of the screened differential metabolites were enriched and topologically analyzed using MetaboAnalyst [15] software package, and the enriched pathways were analyzed using the KEGG Mapper visualization tool for differential metabolite and pathway maps.

Results And Discussion

Analysis by HS-SPME-GC-MS

The volatile component species of *D. officinale* juice in the unfermented group (50 species), single *S. fibuligera* group (40 species), single *L. paracasei* group (50 species), and *S. fibuligera* + *L. paracasei* group (49 species), including alcohols, esters, ketones, acids, phenols, and aldehydes, were detected by HS-SPME-GC-MS (Fig. 1). The alcohol content in the stock solution was 444.806 ± 10.310 $\mu\text{g/mL}$, which significantly decreased to 220.093 ± 21.340 $\mu\text{g/mL}$ with the addition of single *L. paracasei* fermentation, and the total volatile components were reduced by 216.546 ± 35.725 $\mu\text{g/mL}$. The addition of single *S. fibuligera* fermentation significantly increased the alcohol content to 510.999 ± 38.431 $\mu\text{g/mL}$, followed by the contents of linalool, phenyl ethanol, isobutanol, and alpha-pinorexinol. The primary lipid produced was isoamyl formate (236.473 ± 12.868 $\mu\text{g/mL}$). The alcohol content increased to 504.758 ± 77.914 $\mu\text{g/mL}$ in the fermented group of *D. officinale* juice inoculated with *L. paracasei* + *S. fibuligera*, with isoamyl alcohol nearly twice as much as the original solution, which can mellow the taste of *D. officinale* juice and contribute positively to the aroma complexity [16]. This result indicates that the addition of single *S. fibuligera* fermentation and simultaneous inoculation of *L. paracasei* + *S. fibuligera* could increase the alcohol content. *S. fibuligera* did not produce ethyl L(-)-lactate when fermented alone, and the amount of ethyl L(-)-lactate after mixed culture fermentation was three times higher than that after *L. paracasei* fermentation, indicating that the co-culture of these two strains contributed to the metabolic production of ethyl L(-)-lactate. The primary esters in the unfermented *D. officinale* juice were ethyl caproate and isoamyl acetate. The primary esters in the fermented samples were ethyl cinnamate, ethyl caproate, ethyl octanoate, isoamyl formate, and ethyl L(-)-lactate, indicating that the short-chain esters were gradually converted to long-chain esters by microbial metabolism of *D. officinale* juice [17]. Furthermore, fermentation with inoculated strains altered volatile compound distribution (Fig. 1) [18]. Acids play a vital role in fermented beverages as they act as precursors in the synthesis of compounds like esters, alcohols, and methyl ketones. Esters are produced through esterification of organic acids with alcohols or the metabolism of amino acids [19]. However, most organic acids are nonvolatile compounds that cannot be detected by HS-SPME-GCMS. Therefore, the total acid produced by *L. paracasei* and *S. fibuligera* of fermentation metabolism could not be distinguished by volatile acids.

The changes in specific volatile compounds before and after fermentation can be seen through the thermogram, as shown in Fig. 2. The volatile components in group A (unfermented *D. officinale* juice) mainly included 2-octan, 3-chloro-2-methoxypyridine-5-boronic acid, 2,5-dimethyl-2,5-hexanediol, 4-hydroxy-2-butanone, and 1-isopropyl-2-methylbenzene. The volatile components in group B (*D. officinale* juice fermented with *S. fibuligera*) mainly included citronellol, acetophenone, linalool, benzyl alcohol, phenylethylene, geraniol, isoamyl acetate, ethyl 3-phenylpropionate, benzaldehyde, ethyl 2-furoate, ethyl heptanoate, 3,4-dimethyl benzaldehyde, 3-methyl-1-hexanol, cyclooctene, phenyl ethanol, 1-octen-3-ol, heptanol, and dammarone. The volatile components in group C (*D. officinale* juice fermented by *L. paracasei*) mainly included 2,6-Di-tert-butyl-4-methyl phenol, methylheptenone, nonanoic acid, 2-nonanol, terpinolene, 1-nonene, methyl cinnamate, 2(3H)-Furanone, dihydro-5-(2Z)-2-octen-1-yl-, beta-damascenone, acetyl acetone, 4-ethylbenzaldehyde, 2-ethyl hexanol, dimethyl phthalate, 2,4-di-tert butyl phenol, 2-dodecanol, and acetic acid. The volatile components in group C of *D. officinale* juice fermented with the mixed culture of *L. paracasei* and *S. fibuligerae* mainly included ethyl n-hexanoate, ethyl L(-)-lactate, isopentane, and 6,6-trimethyl-(1 theta)-bicyclo[3.1.1]hept-2-en. Alcohols were the primary volatile compounds in this experiment, followed by lipids and ketones, with acids, ketones, alkenes, and phenols being predominant. Alcohols, mainly from the oxidative degradation of polyunsaturated fatty acids, are the secondary lipid oxidation products [20]. Esters are obtained through esterification of low-grade saturated fatty acids and saturated fatty alcohols. Most esters have fruity and floral flavors, which can reduce the pungency of fatty acids and bitterness of amino acids [21]. Ketones are formed mainly by the oxidation of unsaturated fatty acids and the degradation of amino acids, and most ketones have a distinctive light and fruity flavor [22]. The harmonious coexistence of these volatile compounds enriches the characteristic aroma of fermented *D. officinale* juice.

Non-targeted Metabolomics Pca Analysis

Quality control (QC) is essential to obtain reliable and high-quality metabonomic data in mass spectrometry-based metabonomic research [23]. The denser the distribution of QC samples on the PCA analysis plot, the more reliable the data. Figure 3a and b illustrate the PCA scores of QC in the positive and negative ion modes, respectively, and the clustering of QC sample distribution indicates high method stability and good data quality, reproducibility, and reliable results. Figure 4a and b illustrates the PCA scores of different *D. officinale* juice samples in the positive and negative ion modes, respectively. The PCA modeling analysis was performed using uninoculated *D. officinale* juice A as the control group. The PCA results showed that the contribution rates of PC1 and PC2 in the positive ion mode were 34.3% and 15.9%, respectively, and their cumulative contribution rate was 50.2%. Similarly, the contribution rates of PC1 and PC2 in the negative ion mode were 37.2% and 18.8% respectively, and their cumulative contribution rate was 56%. This result indicated that the separation trend between different inoculums of *D. officinale* juice was more prominent, which could reflect the differences of metabolites between different samples [24].

Untargeted Metabolomics Opls-da Analysis

In the PCA model, the differential variables were spread over more principal components due to the influence of relevant variables, which was not conducive to finding the actual differences between the sample groups and screening for valid differential metabolites. The orthogonal projections to latent structures discriminant analysis (OPLS-DA) method can effectively reduce the complexity of the model and enhance the explanatory power of the model without reducing the predictive power of the model to maximize the view of differences between groups [25]. As depicted in Fig. 5, when uninoculated *D. officinale* juice in group A was used as the control group, the aggregation of each group of samples was better, with $R_X^2 = 0.579$, $R_Y^2 = 1.000$ and $Q^2 = 0.820$ in the positive ion mode and $R_X^2 = 0.630$, $R_Y^2 = 0.999$ and $Q^2 = 0.846$ in the negative ion mode, respectively. The Q2 values of the model predictability were close to 1, indicating that the OPLS- DA model is more stable and reliable [26]. The position of all blue Q2 points in the OPLS-DA replacement test diagram was lower than the original blue Q2 points on the right, indicating the reliability and efficacy of the results.

Differential Metabolite Screening

The group A of unfermented *D. officinale* juice was used as the control group, and the single *S. fibuligera* group B, single *L. paracasei* group C, and *S. fibuligera* + *L. paracasei* group D were used as the test groups. As shown in Table 2, when the OPLS-DA first principal component variable importance value projection $VIP > 1$ and P-value < 0.05 , a total of 148 differential metabolites were screened in group B of *S. fibuligera* fermented *D. officinale* juice; of which, 76 were significantly up-regulated and 72 were significantly down-regulated. Similarly, a total of 155 differential metabolites were screened in group C of *L. paracasei* fermented *D. officinale* juice; of which, 75 were significantly up-regulated and 80 were significantly down-regulated. As for the *S. fibuligera* + *L. paracasei* fermented *D. officinale* juice group D, a total of 136 differential metabolites were screened; of which, 75 were significantly up-regulated and 61 were significantly down-regulated. The sample of *D. officinale* juice fermented with *L. paracasei* had the highest number of down-regulated differential metabolites, while the sample of *D. officinale* juice fermented with mixed culture had the lowest number of down-regulated differential metabolites. The transformation of these metabolites significantly changed the flavor of *D. officinale* juice [27].

Table 2
Up-regulation and down-regulation of differential metabolites in the test group

Comparison	Up	Down	Total
B vs. A	76	72	148
C vs. A	75	80	155
D vs. A	75	61	136

In the thermographic cluster analysis (Fig. 6), the redder the color is, the higher the expression amount and abundance are; the bluer the color is, the lower the expression amount and abundance are. It can be seen that the color significantly changed between the test group and control group, and the expression of metabolites varied greatly. The differential metabolites screened from the fermentation group mainly included alcohols, organic acids, amino acids and peptides, and their analogs, nucleic acids and their derivatives, carbohydrates, benzene compounds, terpenoids, alkanes, amines, ketones, and glycosides in 11 classes of compounds. There are 13 kinds of amino acids and peptides and their analogs in Fig. 6a, 12 kinds in b, and 16 kinds in c; the differential metabolites present in a and b but not in c are 5'-deoxy-5'-thionucleosides, flavones, glycosyl compounds, phenylacetaldehyde, porphyrins, purine ribonucleotides, pyrimidine 2'-deoxyribonucleosides, quinoline carboxylic acids, steroids and steroid derivatives, safrole, 3-methyl-L-tyrosine, budesonide methyl ester, (-)-5'-demethylation, (S)-norlaudanine, 9,10-dihydroxystearate, (-)-5'-demethylation, guanosine, Oleuropein aglycone, and Lamiide. The differential metabolites present in a but not in b and c are 2-benzopyrans, pyrethrin, and N7-methylguanosine; the metabolites present in b but not in a and c are 2-phenyl ethanol, trigonelline, L-methionine, and 1,2-Epoxy-p-menth-8-ene. The differential metabolites present in c but not in a and b are anilides, indolyl carboxylic acids, and derivatives, amino catechol, oxalureate, D-glucuronolactone, 2-Oxo-4-phenyl butyric acid, geranyl phosphate, geranyl diphosphate, isopentenyl adenosine, and D-galactose. The above results suggested that the co-culture of *L. paracasei* and *S. fibuligera* could inhibit the expression of some metabolites and enhance the composition of some metabolites. These changes in differential metabolites are essential for flavor formation in fermented beverages [28].

As depicted in Fig. 7, each point in the volcano plot represents a metabolite, and the abscissa represents the logarithm of Log_2 , the quantitative difference multiple of a metabolite in two samples. The ordinate represents the logarithm of $-\log_{10}$ of P value. The larger the absolute value of the horizontal coordinate, the greater the fold difference in the expression of a metabolite between two samples. The larger the value of the vertical coordinate, the more significant the differential expression and the more reliable the differentially expressed metabolites screened. The red points in the graph represent up-regulated differentially expressed metabolites, the blue points represent the down-regulated differentially expressed metabolites, and the grey points represent the metabolites detected without complying to the filtering parameters for screening. The five most significantly up-regulated compounds were 2,3-butanediol, succinic acid, N-acetyl-D-glucosamine, uric acid, and hypoxanthine (Fig. 7a). As depicted in Fig. 7b, the three most significantly up-regulated compounds were wool sterols, succinic acid, and hypoxanthine, and the two most significantly down-regulated compounds were cytosine and methylmalonic acid. As depicted in Fig. 7c, 3-methylxanthine, succinic acid, and hypoxanthine were the three most significantly up-regulated compounds, and cytosine and methylmalonic acid were the two most significantly down-regulated compounds. 2,3-Butanediol is one of the most valuable liquid fuels and can be used as a flavor additive. 2,3-Butanediol has strong UV absorption and antioxidant properties. [29]. Succinic acid is a product and intermediate of anaerobic metabolism of many organisms and tricarboxylic acid cycle. It is used as an intermediate chemical in the production of cosmetics, pharmaceuticals, preservatives, herbicides, detergents, etc. [30]. N-Acetyl-D-Glucosamine (N-AcGA) is a highly sweet reducing

monosaccharide used in the treatment of rheumatoid arthritis. Additionally, it is used as an antioxidant in foods, and an additive in infants and children food, and a sweetener for people with diabetes N [31]. Uric acid, a scavenger of oxygen free radicals, helps maintain blood pressure stability, develops resistance to oxidative stress, and inhibits lipid peroxidation. Hypoxanthine is a natural purine base produced during purine catabolism and then converted into xanthine and uric acid. It can act as a precursor to uric acid and an intermediate in purine degradation by removing reactive oxygen species through the action of xanthine oxidase [32]. Lanosterol, a tetracyclic triterpenoid, is an essential intermediate in cholesterol biosynthesis and raw material for the synthesis of vitamin D and steroid-like hormones [33]. Cytosine is one of the primary bases for nucleic acid synthesis. The down-regulation of cytosine is attributed to the synthesis of nucleic acids and their derivatives. Supplementation of food nucleic acids could significantly improve the immunity of the body, anti-oxidation, influence fat metabolism, and maintain normal intestinal flora [34].

Analysis of differential metabolite KEGG pathways

According to the analysis of KEGG metabolic pathways, a total of 167 metabolic pathways were enriched in the group B vs. A, 40 of which were significant ($p < 0.05$); a total of 170 metabolic pathways were enriched in the group C vs. A, 37 of which were significant ($p < 0.05$); a total of 147 metabolic pathways were enriched in the group D vs. A, 43 of which were significant ($p < 0.05$). The results of the extremely r

The vertical coordinates represent the metabolic pathways, and the horizontal coordinates represent the Impact values enriched into the different metabolic pathways; the higher the value, the higher the contribution of the metabolite detected in that pathway. The color represents the P-value; the redder the P-value, the more minor the P-value; the bluer the P-value, the larger the P-value; the smaller the P-value, the more significant the impact of the detected differential metabolite on that pathway. Among these metabolite pathways, amino acids, organic acids, nucleic acids, were the significant differential metabolites, especially glutamic acid was significantly up-regulated in several metabolic pathways. The decomposition of amino acids affects the significant changes in the flavor of fermentation broth [35]. Organic acids had a positive effect on flavor presentation in sour drinks, with L-malic and citric acid being significantly up-regulated in several metabolic pathways. L-malic and citric acid have soft and intense acidity that enhances the product's mellowness [36].

It can be seen from Fig. 8, 9, and 10 that the differential metabolites of *D. officinale* juice fermented with single *S. fibuligera* significantly differed from those in the *D. officinale* juice fermented with single *L. paracasei* in 7 of the 20 most significant metabolic pathways. There were five differences between the *D. officinale* juice fermented with single *S. fibuligera* and the *D. officinale* juice fermented with *S. fibuligera* + *L. paracasei*, while there were seven differences between the *D. officinale* juice fermented with single *L. paracasei* and the *D. officinale* juice fermented with *S. fibuligera* + *L. paracasei*. However, three pathways existing in the *D. officinale* juice fermented with the mixed culture of *S. fibuligera* + *L. paracasei* were not found in the most significant 20 pathways in the *D. officinale* juice fermented with single *S. fibuligera* and the *D. officinale* juice fermented with single *L. paracasei*, including pentose and glucuronate interconversions, biosynthesis of alkaloids derived from terpenoid and polyketide, and aminobenzoate

degradation. The interconversion of pentose and glucuronic is a metabolic process of carbohydrates and compounds, and terpenoids are the primary active substances with several physiological activities, such as anti-inflammatory, anticancer, anti-bacterial, and anti-viral [37]. Polyketones are mainly used for synthesizing anticancer drugs, lipid-lowering drugs, etc. The degradation of aminobenzoic acid facilitates the synthesis of catechol and 3-methyl catechol.

Analysis Of Secondary Metabolite Synthesis

The addition of strains to ferment *D. officinale* juice facilitated the synthesis of secondary metabolites. As summarized in Table 3, seven kinds of organic acids, four kinds of fatty acids, three kinds of carbohydrates, three kinds of amino acids, three kinds of benzene compounds, two kinds of imidazoles, two kinds of vitamins, two kinds of terpenoids, two kinds of flavonoids, one kind of alkaloid, one kind of ester, and one kind of other metabolite were significantly up-regulated after fermentation. Of the up-regulated metabolites screened, succinic acid, citric acid, and trans-ferulic acid were present in all three sample groups, while 2-hydroxycinnamic acid and salicylic acid were specific to the single *L. paracasei* fermentation group C. Protocatechuic acid and Shikimic acid were significantly up-regulated in the *S. fibuligera* + *L. paracasei* fermentation group D. The log₂FC value of L-malic acid in the mixed fermentation group was the highest, and the down-regulated metabolites, such as methyl jasmonate, jasmonic acid, and sinapic acid, in the mixed fermentation group utilized D-maltose and contributed to the synthesis of D-glucose-1-phosphate, but no amino acid compounds were up-regulated.

Table 3
Analysis of secondary metabolite synthesis in fermented *D. officinale* juice

Classify	Name	m/z	Rt/s	formula	log ₂ FC		
					B vs. A	C vs. A	D vs. A
organic acids	Succinic acid	116.9854	144.7	C ₄ H ₆ O ₄	6.19	4.11	6.17
	Citric acid	191.0198	153	C ₆ H ₈ O ₇	6.02	6.35	3.59
	trans-Ferulic acid	193.0505	335.5	C ₁₀ H ₁₀ O ₄	1.12	1.61	1.28
	2-Hydroxycinnamic acid	165.0552	504.3	C ₉ H ₈ O ₃	-	1.98	-
	Salicylic acid	137.0238	400.9	C ₇ H ₆ O ₃	-	0.2	-
	Protocatechuic acid	153.0192	828.4	C ₇ H ₆ O ₄	-	-	1.83
	Shikimic acid	173.0454	104.9	C ₇ H ₁₀ O ₅	-	-	1.44
fatty acids	L-Malic acid	134.0177	77.5	C ₄ H ₆ O ₅	4.27	2.28	6.3
	Methyl jasmonate	207.1373	634.9	C ₁₃ H ₂₀ O ₃	2.89	6.66	-
	Sinapic acid	207.0648	511.9	C ₁₁ H ₁₂ O ₅	0.88	-	-
	Jasmonic acid	193.1219	706.7	C ₁₂ H ₁₈ O ₃	-	2.33	-
carbohydrates	Trehalose 6-phosphate	421.0747	78.5	C ₁₂ H ₂₃ O ₁₄ P	6.48	7.33	5.8
	D-Maltose	342.1391	141.9	C ₁₂ H ₂₂ O ₁₁	2.2	2.38	-
	D-Glucose 1-phosphate	261.0361	90	C ₆ H ₁₃ O ₉ P	-	-	2.41
amino acids	Pipecolic acid	130.0854	142.6	C ₆ H ₁₁ NO ₂	2.71	-	-
	L-Glutamic acid	130.0486	167.9	C ₅ H ₉ NO ₄	2.22	0.67	-
	L-Aspartic acid	132.0304	81.5	C ₄ H ₇ NO ₄	-	2.86	-
benzene compounds	5-Hydroxyconiferyl alcohol	179.0709	286.6	C ₁₀ H ₁₂ O ₄	1.29	-	-
	(E)-3-(4-Hydroxyphenyl)-2-propenal	131.0488	650.8	C ₉ H ₈ O ₂	-	3.59	-

Classify	Name	m/z	Rt/s	formula	log ₂ FC		
					B vs. A	C vs. A	D vs. A
	Coniferyl alcohol	181.0862	457.3	C ₁₀ H ₁₂ O ₃	-	0.6	0.96
imidazoles	3-Methylxanthine	166.0496	336.9	C ₆ H ₆ N ₄ O ₂	6.94	7.3	7.05
	1-Methylxanthine	166.0497	353.3	C ₆ H ₆ N ₄ O ₂	2.43	-	2.39
vitamins	alpha-Tocopherol	430.2402	495.7	C ₂₉ H ₅₀ O ₂	6.9	6.81	6.45
	Pantothenic acid	218.1036	195.8	C ₉ H ₁₇ NO ₅	2.57	2.63	-
terpenoids	Lanosterin	425.2556	814.5	C ₃₀ H ₅₀ O	5.28	5.77	-
	Sclareol	291.2669	790.6	C ₂₀ H ₃₆ O ₂	1.89	2.01	1.74
flavonoids	Genistein	271.2257	686	C ₁₅ H ₁₀ O ₅	4.71	4.41	4.68
	Naringenin	271.061	647.1	C ₁₅ H ₁₂ O ₅	1.66	2.14	-
alkaloid	Ecgonine methyl ester	182.1171	693.7	C ₁₀ H ₁₇ NO ₃	1.03	1.14	-
ester	Benzoate	121.0294	524.3	C ₇ H ₆ O ₂	-	-	2.95
other	Geranyl diphosphate	314.0903	250.4	C ₁₀ H ₂₀ O ₇ P ₂	-	-	1.91

Analysis Of Key Metabolite Pathways In The Mixed Culture Groups

The metabolic pathways of the differential metabolites in the fermented *D. officinale* juice from *S. fibuligera* + *L. paracasei* were analyzed. In Fig. 11, the blue dot represents the pathway, and other dots represent the metabolites. The size of the pathway dot represents the number of metabolites connected to it. The metabolite dot shows the size of log₂FC value through the gradient color. A total of ten key metabolic pathways were obtained, including linoleic acid metabolism, phenylpropanoid biosynthesis, cAMP signaling pathway, pentose and glucuronate interconversions, glucagon signaling pathway, taste transduction, citrate cycle (TCA cycle), central carbon metabolism in cancer, synaptic vesicle cycle, and ABC transporters. As depicted in Fig. 11, 23 kinds of metabolites were significantly up-regulated in the ten critical metabolic pathways, namely 9,10-DHOME, D-Xylono-1,5-lactone, gamma-minobutyric acid, genistein, succinic acid, trans-ferulic acid, L-malic acid, citric acid, coniferyl alcohol, protocatechuic acid, shikimic acid, D-glucose 1-phosphate, biotin, inosine, D-xylitol, L-arabinose, 9,10-dihydroxy-12,13-epoxyoctadecanoate, 9,10-epoxyoctadecenoic acid, D-lyxonate, 2'-deoxyguanosine, D-arabinol, and D-

gulucronic acid. While 11 kinds of acid metabolites were significantly up-regulated, exerting a positive effect on *D. officinale* juice flavor formation.

Conclusions

In this study, the headspace solid-phase microextraction-gas combination technology was used to analyze the changes in the volatile components before and after fermentation of *D. officinale* juices. The differential metabolites were screened using the non-targeted metabolisms of liquid chromatography-mass spectrometry combined with multiple statistics through analytical methods. The results showed that the alcohol contents in the *S. fibuligera* group before fermentation and after fermentation were 444.806 ± 10.310 $\mu\text{g/mL}$ and 510.999 ± 38.431 $\mu\text{g/mL}$, respectively. While the alcohol content in the fermentation broth group inoculated with the co-culture of *L. paracasei* + *S. fibuligera* was 504.758 ± 77.914 $\mu\text{g/mL}$. The contents of phenylene alcohol and isobutanol showed a significantly increasing trend. Additionally, the content of ethyl L(-)-lactate in the *L. paracasei* + *S. fibuligera* fermentation group D was higher than that of the *L. paracasei* monobacterial fermentation group C and *S. fibuligera* monobacterial fermentation group B, indicating that the co-culture of the *L. paracasei* + *S. fibuligera* was conducive to ethyl L(-)-lactate in the *D. officinale* juices. Experiments have shown that *D. officinale* juices fermented by *L. paracasei* + *S. fibuligera* can prevent the downregulation of some metabolites, and can also enhance Pentose and glucuronate interconversions, Biosynthesis of alkaloid, the significance of the three metabolic pathways derived from terpenoid and polyketide and Aminobenzoate degradation, which are beneficial to biological activities such as terpenes, polyketones and phenols. Synthesis of substances, rich in the flavor composition of *D. officinale* juices. Overall, the study results demonstrate that the fermentation of *D. officinale* juices by simultaneous inoculation of *L. paracasei* and *S. fibuligera* could promote the flavor coordination between fermented products and provide a theoretical basis for further study of the flavour of *D. officinale* juices and the functional exploration of fermentation metabolites.

Abbreviations

D. officinale

Dendrobium officinale

Lactobacillus paracasei

L. paracasei

Saccharomycopsis fibuligera

S. fibuligera

Declarations

Availability of data and materials

All data generated or analyzed during this study have been included in this published article and its supplementary information files.

Statement

The experimental material comes from Guizhou Qiansheng Taijiang Poverty Alleviation Industry Development Co., LTD., which is located in Guizhou Province, China. The experimental material is legal and compliant. It is in accordance with the relevant guidelines of the journal.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

Ethics approval and consent to participate This article does not contain any studies with human or animal subjects.

Consent for publication Not applicable.

Competing interests The authors declare that they have no conflict of interest.

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Authors' contributions

Xiaoye Luo and Wanlin Liu wrote the original draft manuscript and conducted the experiments; WU Huang, Yanan Su, and Linling LI analyzed the data; Shuyi Qiu conceive and designed the experiments. All authors contributed to the article and approved the submitted version. The authors read and approved the final manuscript.

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Figures

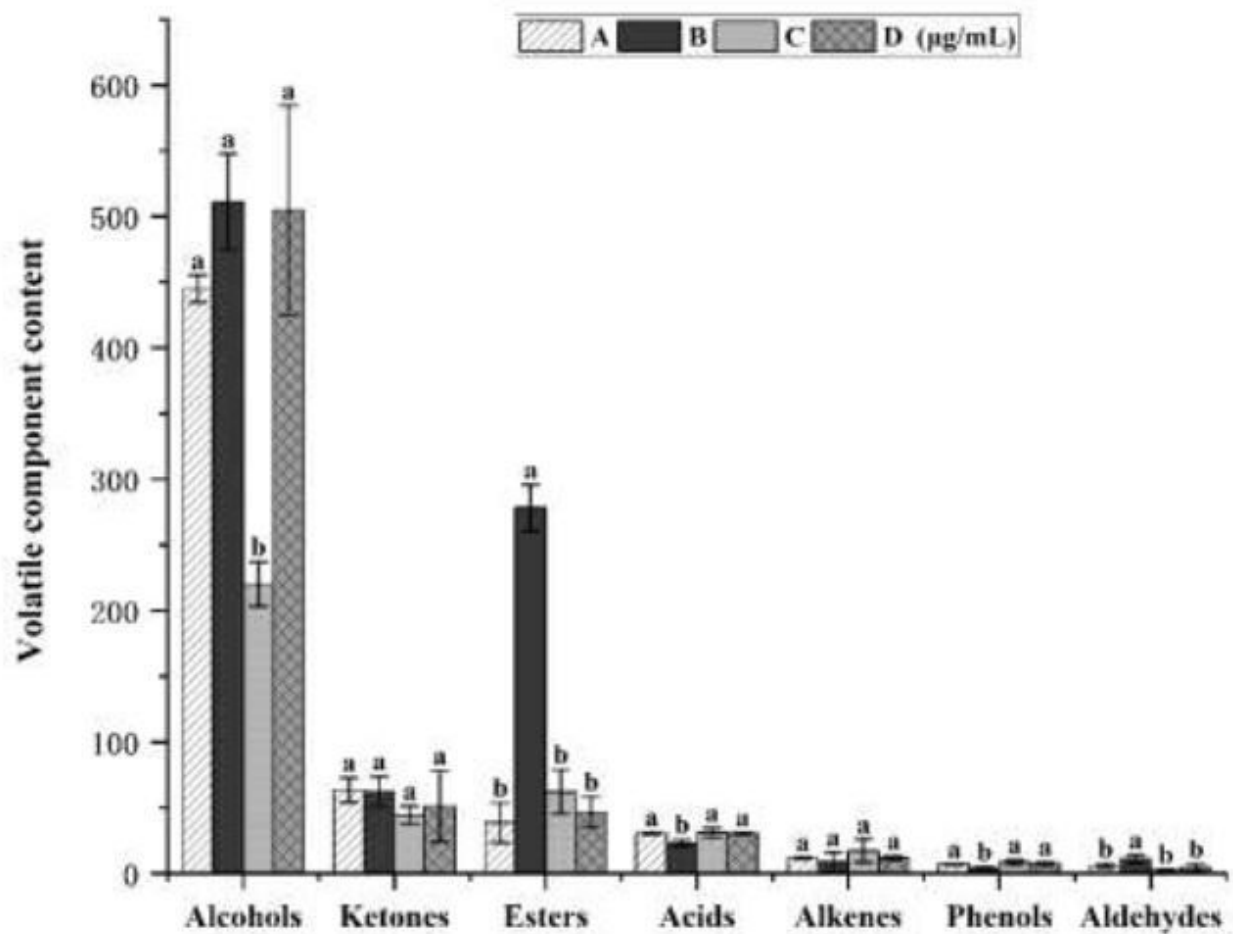


Figure 1

Relative volatile components of *D. officinale* juice before and after fermentation (The unfermented *D. officinale* juice (A), *S. fibuligera* fermented *D. officinale* juice (B), *L. paracasei* fermented *D. officinale* juice (C), *L. paracasei* + *S. fibuligera* fermented *D. officinale* juice (D))

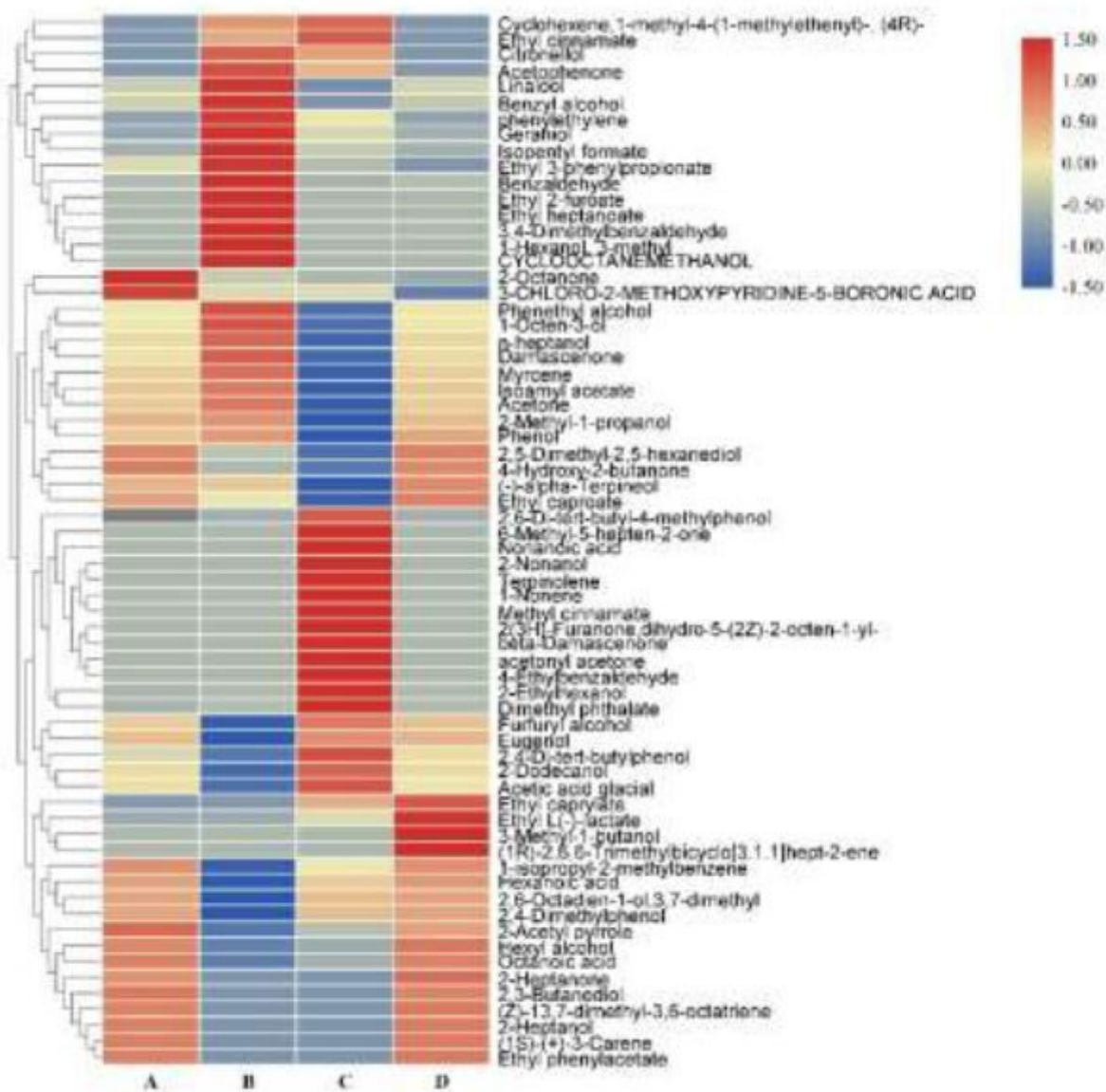


Figure 2

Heat map of the volatile components in *D. officinale* juice with different inoculations

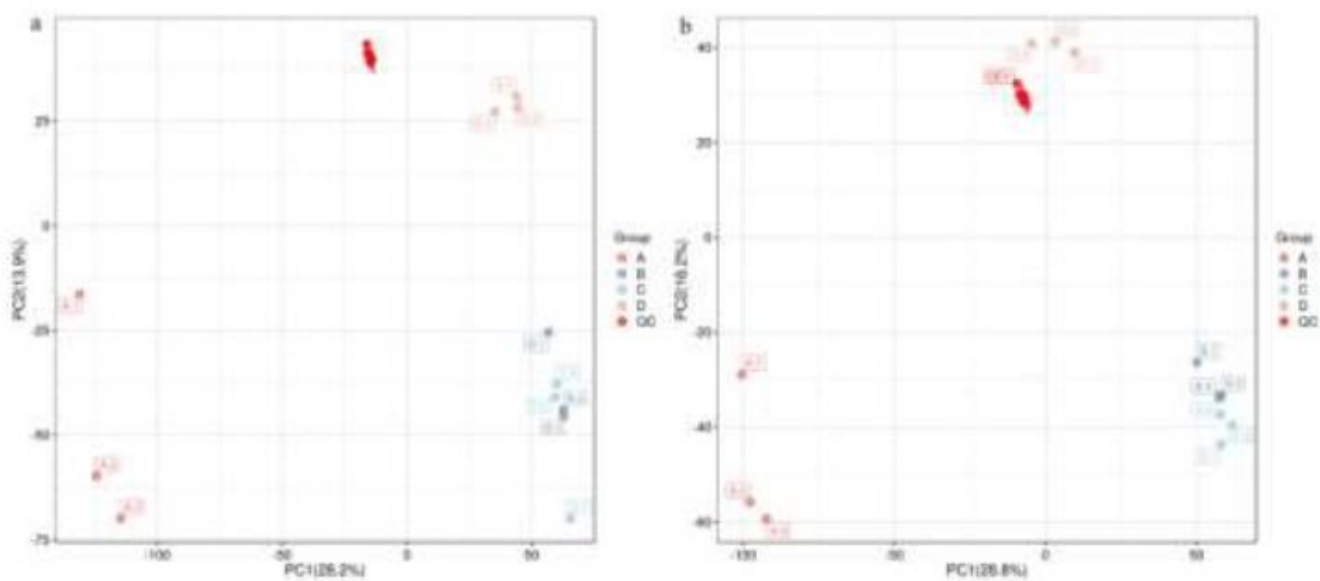


Figure 3

PCA score plots for QC samples in the positive ion mode (a) and negative ion mode (b)

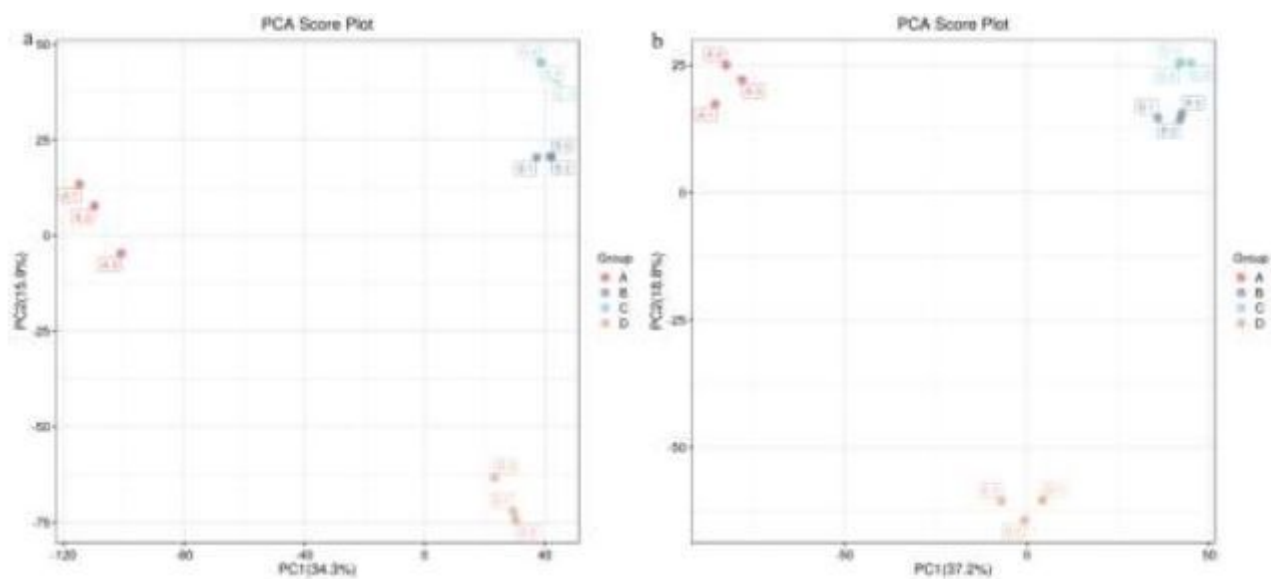


Figure 4

PCA scores of different *D. officinale* juice samples in the positive ion mode (a) and negative ion mode (b)

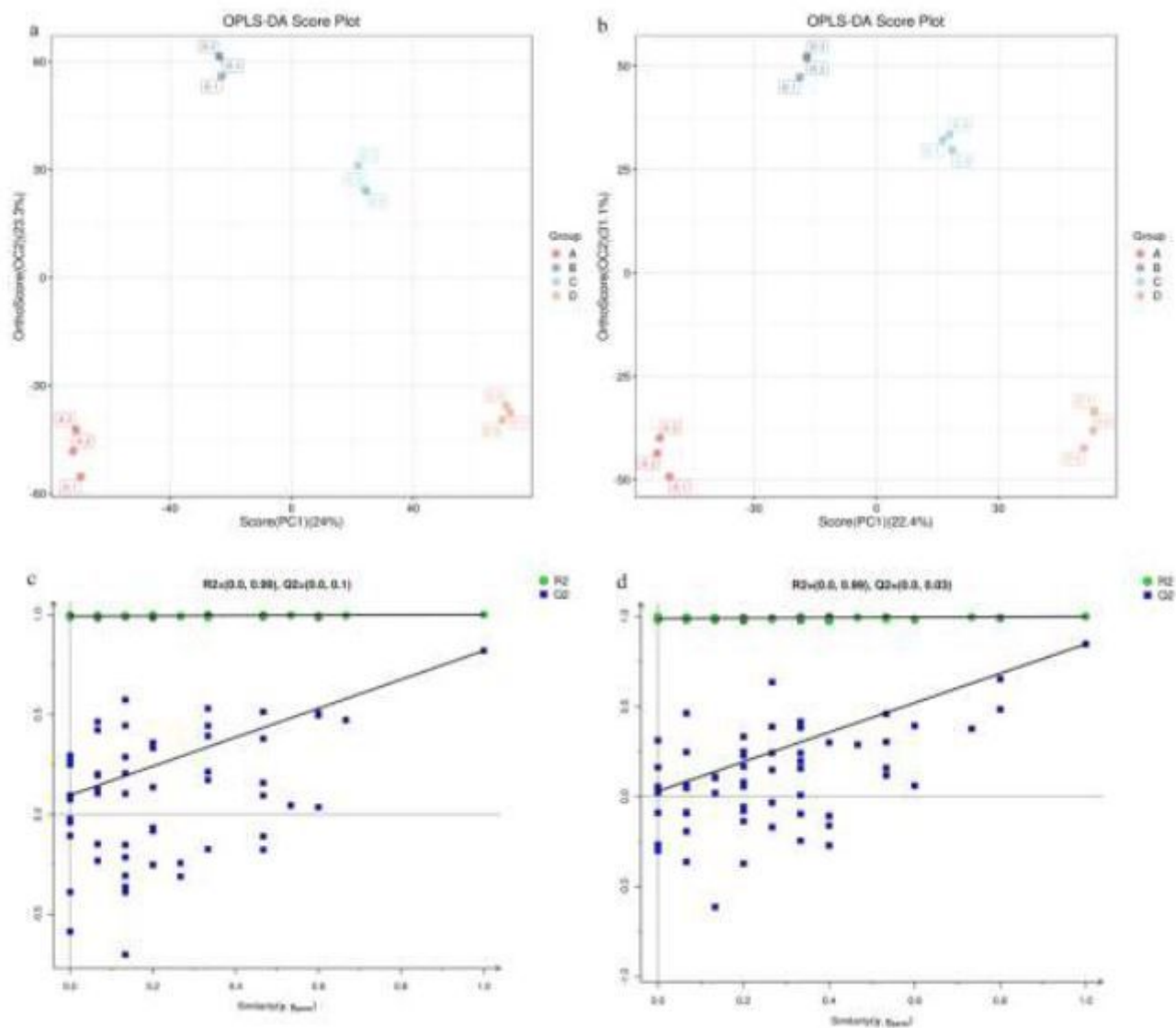


Figure 5

Plots of OPLS-DA scores for different groups of metabolites in the positive and negative ion modes a, b, and substitution tests c, d

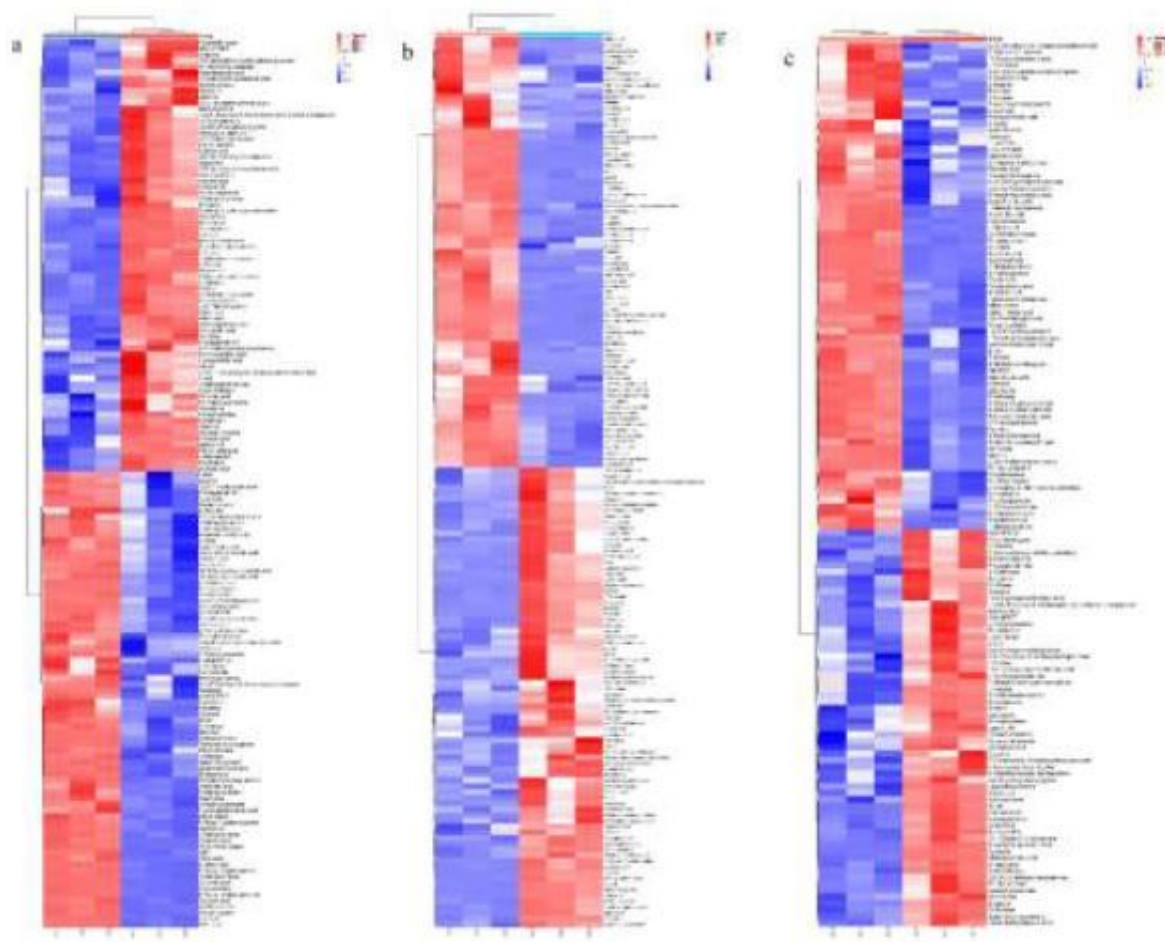


Figure 6

a shows the heat map of BvsA differential metabolites, b shows the heat map of CvsA differential metabolites, c shows the heat map of DvsA differential metabolites

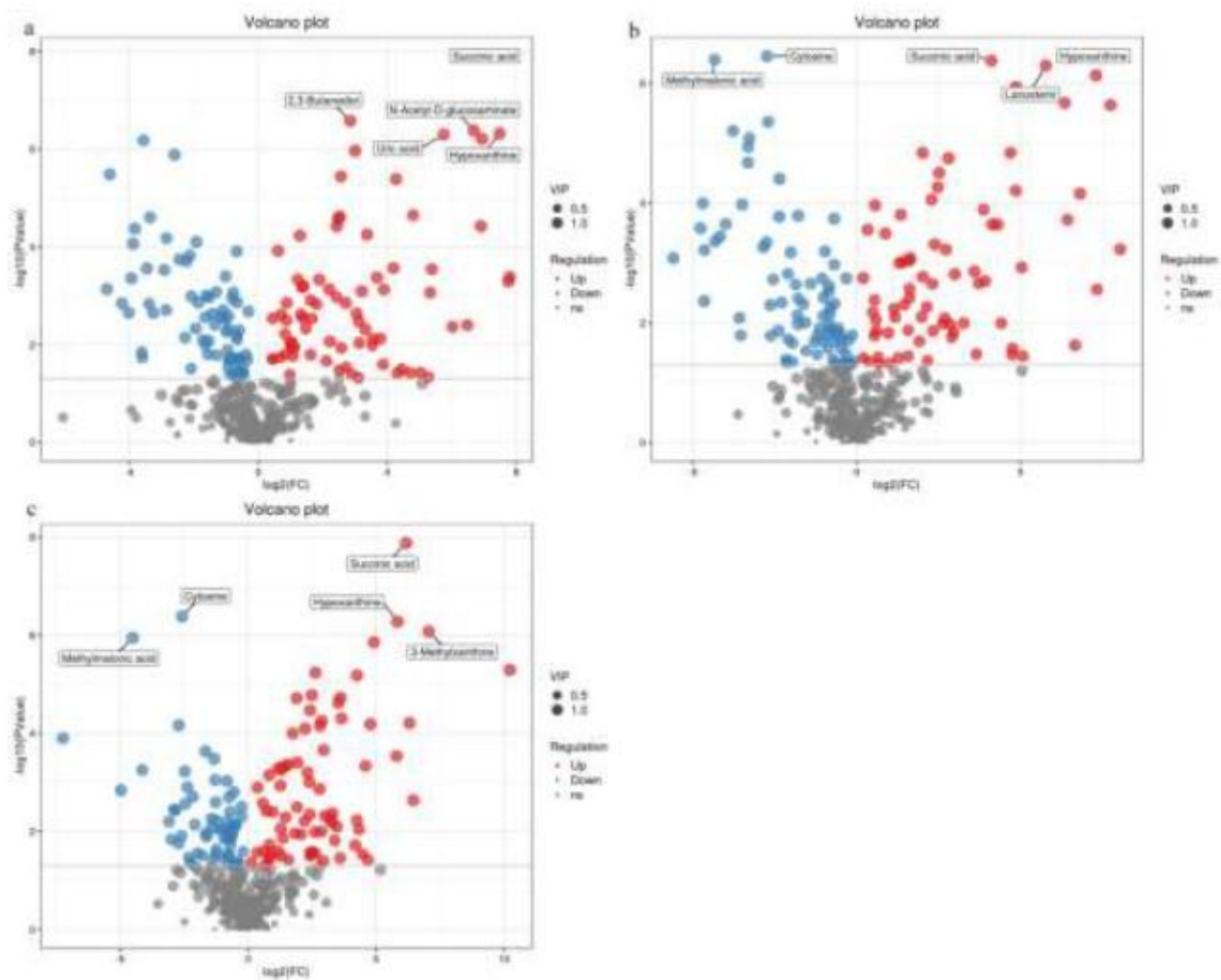


Figure 7

a is for the compound volcanoes of B vs. A, b is for the compound volcanoes of B vs. A, c is for the compound volcanoes of C vs. A

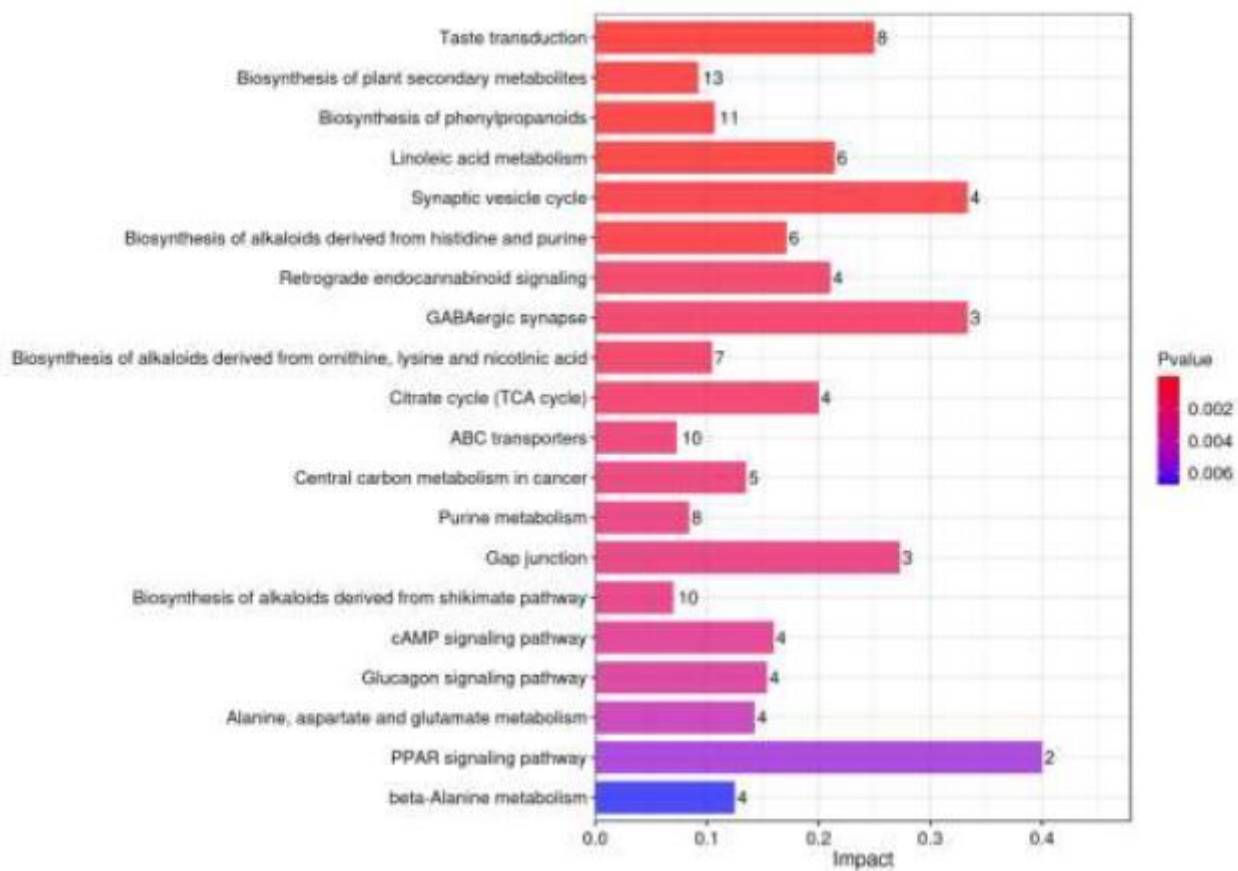


Figure 8

Histogram of factors influencing the B vs. A metabolic pathway

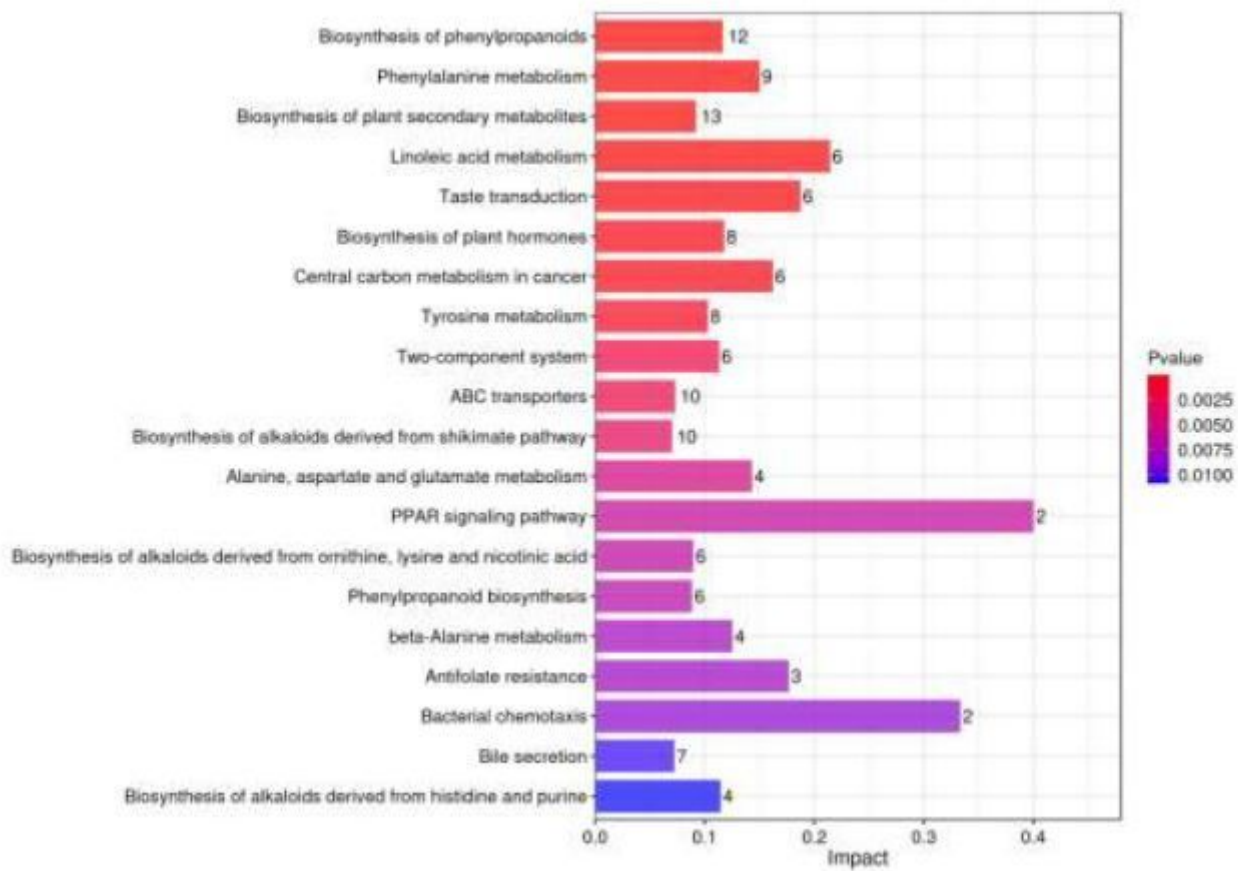


Figure 9

Histogram of factors influencing the C vs. A metabolic pathway

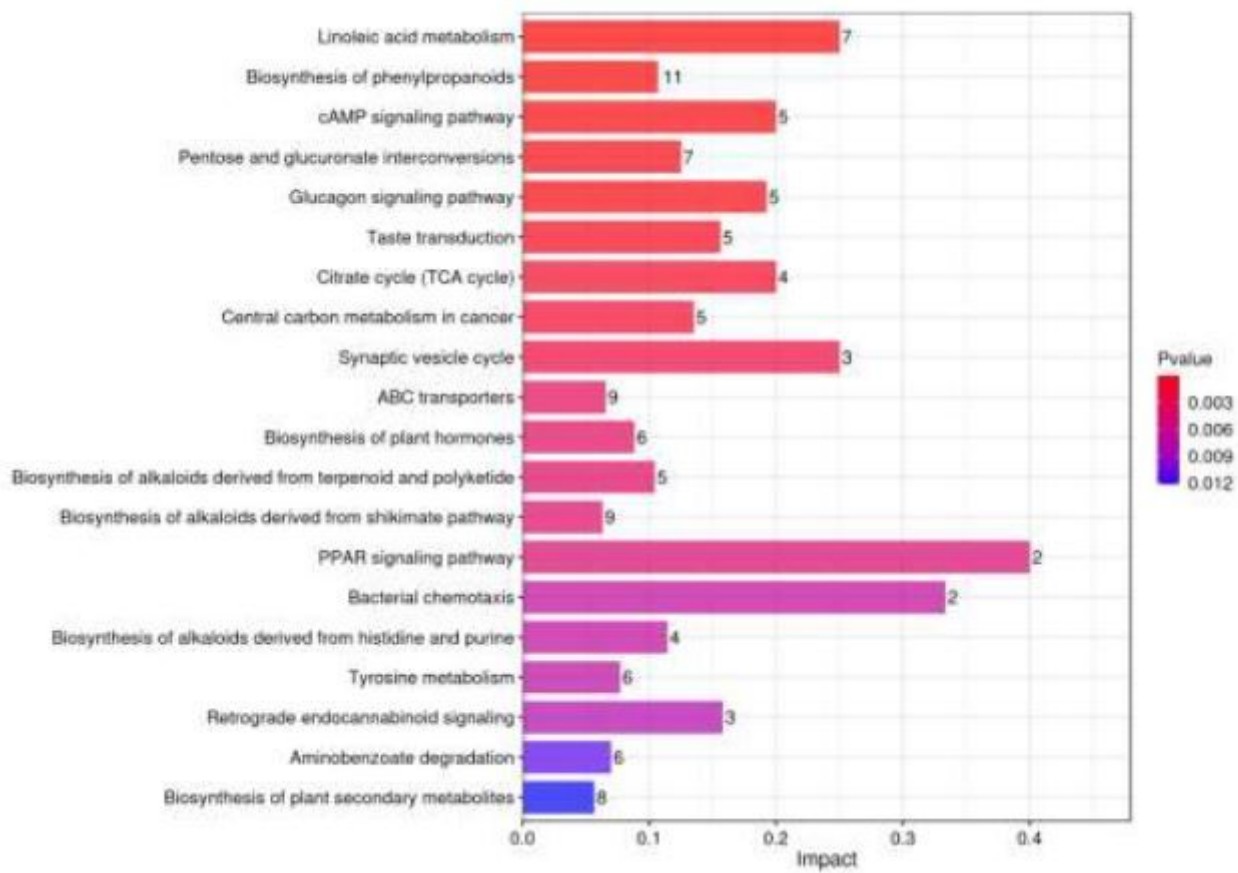


Figure 10

Histogram of factors influencing the D vs. A metabolic pathway

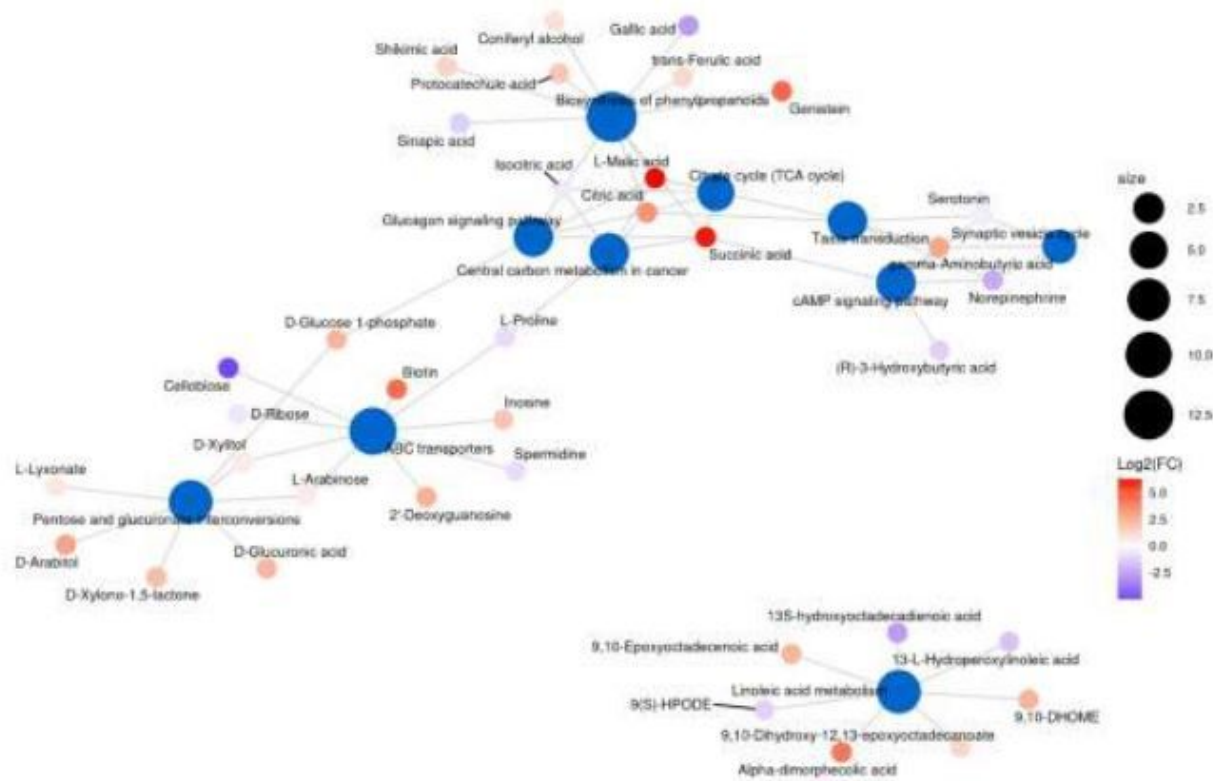


Figure 11

Differential metabolite network diagram

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GCMSdata.xlsx](#)
- [LCMSdata.xlsx](#)