

# Analysis of Primary Metabolites of *Morchella* Fruit Bodies And Mycelium Based On Widely Targeted Metabolomics

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## Research Article

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# Abstract

*Morchella* is a kind of medicinal and edible homologous fungus that is rich in multiple metabolites. The metabolites from *Morchella* are a kind of essential substance because of their biological activities. In this study, *Morchella* fruit bodies and mycelium were selected to identify their metabolites. The primary metabolites of the two experimental groups were analyzed using a method of widely targeted metabolomics based on UPLC-ESI-MS/MS. A total of 354 different metabolites, including 188 upregulated metabolites and 166 downregulated metabolites, were characterized. Further, the main 20 metabolic pathways of the metabolites were analyzed. The first 9 ones are tyrosine metabolites, thyroid hormone biosynthetic pathway, phenylalanine metabolites, linoleic acid synthetic pathway, glycerophosphate metabolic pathway, choline in tumors, methyl butyl metabolites, arginine synthetic pathway, arginine, arginine and proline metabolites. This study provides theoretical basis for the analysis of metabolic pathway of *Morchella* fruit bodies and mycelium that serving for further research of their medicinal mechanism and effective components.

## 1. Introduction

*Morchella* is well known for its high nutritional value and medicinal benefits (Kanwal and Reddy 2014). The fruit bodies and mycelium of *Morchella* are rich in nutrients, including protein, fat, carbohydrates, crude fiber, riboflavin, niacin, folic acid, vitamins and other components (Enkhjargal et al. 2011). *Morchella* is sweet in taste and can be used as medicine (Harpreet et al. 2011) which is famous for its antioxidant properties and shows extremely high medicinal value (B?Ckerman and Maliranta 2013). *Morchella* contains polysaccharides, enzymes, pyrone antibiotics, fatty acids and other chemical components (Gursoy et al. 2009). Modern medical research has shown that *Morchella* has various biology activities such as lowering blood lipids, regulating body immunity, anti-fatigue, protecting liver, anti-virus, inhibiting tumors, and reducing the side effects caused by radiotherapy and chemotherapy (Hai-Bin 2019). However, there was a lack of a thorough and dynamic evaluation of the identified metabolites in *Morchella* fruit bodies and mycelium.

Metabolomics is a rapidly emerging discipline in biomics and is an important part of systems biology (Widiarsih et al. 2021). Metabolomics research uses high-throughput chemical analysis technology to perform qualitative and quantitative analysis of small molecule metabolites in biological samples (Lin et al. 2011). Previous studies have shown that metabolomics is widely used in nutrition science, disease diagnosis, toxicology, plant metabolism and response mechanism and other aspects (Muazu et al. 2021). Wang et al. (Wang et al. 2018) identified the nutrients in black sesame seeds and related metabolites that play a role in traditional Chinese medicine based on extensively targeted metabolomics technology. Ho et al. (Ho et al. 2018) used metabolomics technology to identify 6 compounds with antibacterial activity from black walnut. Based on the quantitative analysis of metabolites, metabolomics can be used for the analysis of metabolic pathways or metabolic networks, the basic research of metabolism of the macroscopic phenotypic phenomena of different organisms, and

the metabolites of different diseases, drugs and other physical, chemical or pathogenic organisms(Zhou et al. 2020).

For the past few years, UPLC-ESI-MS/MS-based, widely targeted metabolome has become very popular in the field of analysis and identification of plant metabolites due to its advantages of high throughput, fast separation, high sensitivity, and wide coverage. UPLC-ESI-MS/MS-based widely targeted metabolome becomes an effective tool to deeply research secondary metabolites (Wang et al. 2019).

Up to now, researchers have reported that the types of bioactive compounds in fungi are often different. However, there is a lack of research about the use of widely targeted metabolome to analyze the metabolic components of *Morchella* fruiting bodies and *Morchella* mycelium. In this study, we selected *Morchella* fruit bodies and mycelium as research materials, using ultra-high performance liquid chromatography tandem mass spectrometry technology to detect the types of metabolites of them. By means of comparing and analyzing their differences, it characterized the chemical substances in *Morchella* fruit bodies and mycelium from the perspective of different metabolites, providing reference for research on functional ingredients. Our study may be also help to understand the biological processes and mechanisms of the fruit bodies and mycelium more intuitively and effectively(Nadia et al. 2015) and might facilitate a deeply understanding of metabolites between *Morchella* fruit bodies and mycelium and provide a reference for their sufficient utilization in the future.

## 2. Materials And Methods

### 2.1. Materials

*Morchella* fruit bodies: Collected from the planation base of Shenyang Agricultural University.

*Morchella* mycelium: The *Morchella* strain was obtained from the Academy of Biological Science and Technology, Shenyang Agricultural University (Shenyang, China). *Morchella* mycelia were cultured on Potato Dextrose Agar medium in flasks at 28°C for 7 days in a rotary shaker at 120 rpm. The suspension was centrifuged and lyophilised to obtain the best fermented *Morchella* mycelium.

### 2.2 Sample Preparation

Thaw the sample on ice. Take 50 mg of the sample and homogenize it with 1000 uL of ice-cold methanol/water (70%, v/v). Add cold steel balls to the mixture and homogenate for at 30Hz for 3 min. Whirl the mixture for 1 min, and then centrifuge it with 12,000 rpm at 4°C for 10 min. The supernatant collected will be used for LC-MS/MS analysis(Xian et al. 2008).

### 2.3 Analysis by LC-MS/MS

The sample extracts were analyzed with an LC-ESI-MS/MS system (UPLC, Shim-pack UFLC SHIMADZU CBM A system, <https://www.shimadzu.com/>; MS, QTRAP® System, <https://sciex.com/>). The analytical conditions were as follows: UPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 µm, 2.1 mm\*100

mm); column temperature, 40 °C; flow rate, 0.4 mL/min; injection volume, 2µL; solvent system, water (0.04% acetic acid): acetonitrile (0.04% acetic acid); gradient program, 95:5 V/V at 0 min, 5:95 V/V at 11.0 min, 5:95 V/V at 12.0 min, 95:5 V/V at 12.1 min, 95:5 V/V at 14.0 min.

LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (QTRAP), QTRAP® LC-MS/MS System, equipped with an ESI Turbo Ion-Spray interface, operating in positive and negative ion mode and controlled by Analyst 1.6.3 software (Sciex). The ESI source operation parameters were as follows: source temperature 500 °C; ion spray voltage (IS) 5500 V (positive), -4500 V (negative); ion source gas I (GSI), gas II (GSII), curtain gas (CUR) were set at 55, 60, and 25.0 psi, respectively; the collision gas (CAD) was high. Instrument tuning and mass calibration were performed with 10 and 100 µmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period(Oh et al. 2011) .

## 2.4 Quality control analysis

Quality control samples ( QC ) were prepared by mixing sample extracts and were used to monitor the repeatability of analytical samples under the same processing method. In the process of instrumental analysis, a quality control sample was inserted into every 10 test analysis samples to monitor the repeatability of the analysis process(Bürger et al. 2012).

## 2.5 Data analysis

Qualitative analysis of metabolites were based on MVDB V2.0 database of Maiwei Biotechnology Co., Ltd. and metabolite information in public databases. The primary and secondary mass spectrometry analysis were based on the existing MassBank, KNAPSACK, HMDB and METLIN mass spectrometry databases(Isoherranen et al. 2009). A triple quadrupole mass spectrometry multiple reaction monitoring mode ( MRM ) was used for quantitative analysis of metabolites. After obtaining the metabolite data of different samples, use the software Analyst 1.6.3 to perform mass spectrometry qualitative and quantitative analysis, including baseline filtering, peak identification, integration, retention time correction, peak alignment, and mass spectrometry fragment attribution analysis(Bessa et al. 2013). The data was normalized and annotated based on the obtained retention time, mass-to-severity ratio and peak intensity. At the same time, most substances were further confirmed with standard products. Multi-dimensional statistical analysis was used to establish a reliable mathematical model to analyze metabolites(Markandan et al. 2021). First, use principal component analysis (PCA ) of unsupervised pattern recognition to analyze the detected metabolites to get a preliminary understanding of the overall metabolite differences between samples in each group and the degree of variability between samples within the group. Then use partial least squares-discriminant analysis ( PLS-DA ) with supervised pattern recognition to distinguish the overall differences in metabolites between groups to find different metabolites(Cozzolino et al. 2014). Use orthogonal partial least squares discriminant analysis ( OPLS-DA ) to remove irrelevant differences to screen the difference variables to find the differences between the samples in each group, and the variable weight value (Variable importance in projection, VIP ) greater

than 1 was considered to be a difference variable (Boccard and Rutledge 2013). Evaluation of PLA-DA and OPLS-DA predictive model parameters were  $R^2_X$ ,  $R^2_Y$  and  $Q^2$ . The closer the 3 indicators were to 1, the more stable and reliable the model was. Use multi-dimensional statistics VIP value (VIP > 1), single-dimensional statistics (P < 0.05) and multiple of difference (fold change) to screen different metabolites. The multiple of difference was transformed by Log2, and select VIP > 1, P < 0.05, Log2FC  $\geq$  2 or metabolites with Log2FC  $\leq$  0.5 as differential metabolites (Notararigo et al. 2021).

## 3. Results

### 3.1 Overall analysis of metabolic components

Based on Maiwei (Wuhan) self-built metabolite database and related mass spectrometry database, the multi-peak map of MRM metabolites was detected, the characteristic ions of each substance were screened through the triple quadrupole rod, and the signal intensity of the characteristic ions was obtained in the detector (CPS). Use MultiaQuant software to open the sample offline mass spectrometry file, and perform qualitative and quantitative analysis of the main metabolites. In *Morchella* fruit bodies and mycelium, 34 types of 610 metabolites were identified (Tab.1).

Table 1  
Primary metabolites identified in *Morchella* fruit bodies and mycelium

Category of Metabolites	Total Number of Metabolites (species)	Proportion ( % )
Organic acids and their derivatives	122	20.0
Amino acids and their metabolites	103	16.8
Nucleotides and their metabolites	61	10.0
Benzene and its derivatives	61	10.0
Carbohydrates and their metabolites	42	6.9
Lipids and other fatty acids	32	5.2
Indole and its derivatives	18	2.9
Coenzymes and vitamins	18	2.9
Pyridine and its derivatives	18	2.8
Phenols and their derivatives	16	2.6
Lipids and other phospholipids	16	2.6
Fatty acyl	14	2.2
Oxidized lipid	12	1.9
Lipid	9	1.4
Heterocyclic compound	9	1.4
Ketones	6	0.9
Polyamine	5	0.8
Pteridine and its derivatives	5	0.8
Benzoic acid and its derivatives	5	0.8
Carnitines	4	0.6
Other	34	4.2

## 3.2 Metabonomics difference analysis of *Morchella* fruit bodies and mycelium

### 3.2.1 PCA results

PCA results can generally reflect the metabolite differences between the two groups of samples. Through the principal component analysis (PCA) of the samples, the degree of variability between the groups of *Morchella* fruit bodies and mycelium samples and between the samples within the group could be discriminated. The results contained three principal components (PCs), of which the contribution rate of PC1 (99.79%), PC2 (0.15%) and PC3 (0.03%) respectively. The two groups of samples showed a clear separation trend on the graph (Fig. 1).

### 3.2.2 OPLS-DA results

Although the PCA analysis method can effectively extract the main information, it is not sensitive to less correlated variables. The PLS-DA can maximize the distinction between groups, which is conducive to finding different metabolites<sup>(Peng et al. 2018)</sup>. Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) combined orthogonal signal correction (OSC) and PLS-DA methods to screen difference variables by removing irrelevant differences. The OPLS-DA model (Fig. 2) of 610 metabolites set of data analysis showed that the sample distribution of *Morchella* fruit bodies was on the right side of the confidence interval, while mycelium samples was on the left side. The difference effect of the two samples was often obvious. Two principal components were analysed by OPLS-DA, of which the contribution rate were 98.6% and 0.623%.  $R^2X=0.992$ ,  $R^2Y=1$ ,  $Q^2=1$  (Fig. 3), showing that the grouping model had a strong interpretation and prediction ability, and the clustering results were reliable. The effect was better than the PCA model. Perform permutation verification to OPLS-DA (n=200, that is, conduct 200 permutation experiments).

### 3.2.3 Identification of Differential Metabolites

In the obtained multivariate analysis of the variable importance in project (VIP) of the OPLS-DA model, VIP value represents the difference between the groups of the corresponding metabolites in the classification of each group of samples in the model. The intensity of the impact is considered to be significant generally for metabolites with  $VIP \geq 1$ . The metabolites with differences between the two types of samples were initially screened, and then select the metabolites with  $VIP \geq 1$ . *Morchella* fruit bodies and mycelium were screened to obtain a difference metabolite 32 class 354 species. Overall, the different metabolic components (354 kinds) of *Morchella* fruit bodies and mycelium accounted for 58.03% of total metabolic components (610 kinds), indicating the *Morchella* fruit bodies and the *Morchella* mycelial metabolites were significantly different. Among them, there were five categories of organic acids and derivatives, amino acids and their metabolites, nucleotides and their metabolites, benzene and their derivatives, and carbohydrates and their metabolites, accounting for 20.0%, 16.8%, 10.0%, 10.0% and 6.9% respectively.

## 3.3 Differential metabolite analysis

### 3.3.1 Analysis of main differences in metabolic components

Comparing the difference fold change of the quantitative information of the metabolic components in the fruit bodies and mycelium of *Morchella* and processing it by log<sub>2</sub>, the top 20 differentially expressed metabolic components are shown in Fig. 4. The relative content of Trans-4-Hydroxy-L-Proline, N- $\alpha$ -Acetyl-L-asparagine, 3-Iodo-L-Tyrosine, L-phenylalanyl-L-proline, N-Acetyl-L-alanine, N-amidino-L-aspartic acid, 3-Ketone-sphingosine, N-Phenylacetyl-glycine, 3-Methoxytyramine, and Ellagic acid were significantly high in mycelium. The relative content of N-methyl-ephedrine, 2-Furoylglycine, Herniarin, Caffeic Acid, Indolelactic acid, 3,5-Dimethoxy-4-Hydroxycinnamic Acid, 3-Hydroxy-DL-kynurenine, Xanthosine, Melatonin, and Lysopc 14:0 were significantly high in the fruit bodies. N-Methyl-ephedrine is one of the main components of Chinese herbal medicine Ephedra, similar to ephedrine hydrochloride, with an effect of expanding the bronchus (Wei et al. 2009). Without the excitatory center of ephedrine co-acid salt, it has stronger anti-allergic and antitussive effects, and can accelerate heart rate and raise blood pressure (Holzgrabe et al. 2015), which is suitable for the treatment of influenza, bronchial wheezing, allergic reactions and other pathogens (Zhang et al. 2013). The mycelium and fruit bodies are rich in different active ingredients, mainly because of the difference between their development in liquid fermentation and solid cultivation, leading to different metabolites.

### 3.3.2 Differential metabolite Volcanic maps analysis

In order to facilitate the observation of the changes of metabolites, normalize the metabolites with significant differences and draw a volcanic map. Differential metabolites (188 upregulated and 166 downregulated) were detected (Fig. 5), representing 53.11% and 46.89% (Tab. 2).

Table 2  
Statistical table of the number of differential metabolites

groupName	totalSigMetabolites	Down-regulated	Up-regulated
Fruit_bodies_of_morchella_vs_	354	166	188
Fermented_mycelia_of_morchella			

### 3.4 Pathway analysis of differential metabolites

The results of pathway enrichment analysis of differential metabolites through the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (Zhang et al. 2017) (Fig. 6) showed that the identified 354 significantly different metabolites were mainly distributed in 20 metabolic pathways. The first nine pathways with the largest number of differential metabolites were tyrosine metabolism; thyroid hormone synthesis; benzene Alanine metabolite synthesis pathway (phenylalanine metabolism); linoleic acid metabolism synthesis pathway (linoleic acid metabolism); glycerophospholipid metabolism (glycerophospholipid metabolism); choline metabolism in cancer (choline metabolism in cancer); methyl butyrate metabolite synthesis pathway (butanoate metabolism); arginine synthetic pathway (arginine biosynthesis); arginine and proline metabolites biosynthetic pathway (arginine and proline metabolism).



## 4. Discussion

*Morchella* is an important kind of fungi that exhibits various bioactivity due to its variety of metabolites. However, its metabolites and main pathways have not been previously investigated. The utilization rate of *Morchella* is extremely inadequate. Thus, this study aimed to provide a theoretical basis for the utilization of *Morchella*. In this study, the primary metabolites of *Morchella* were comprehensively analyzed. The analysis of the experimental data showed that 354 different compounds were reported for the first time. The first 10 richest compounds in the fruit bodies and mycelium of *Morchella* were different and exhibited diverse efficacy.

Comparison groups showed that the anti-4-hydroxyL-proline was higher in mycelium, which mainly used as flavor agent, nutritional intensifier, flavor material and biochemical reagents in present study. Aromatic amino acids, including phenylalanine, tyrosine and tryptophan, were important essential amino acids in organisms for their important biological functions. 3-iodide tyrosine and N-acetyl-L-alanine were richer in mycelium which could be widely used in fields of medicine, food and feed(Chen et al. 2019). N-methyl ephedrine rich in *Morchella* fruit bodies was one of the main Chinese medicinal herbs, that exhibited the effects of dilating bronchial lines and a stronger anti-allergy and antitussive. They were suitable for the treatment of influenza, bronchial wheezing, allergic reaction and other bacteria(A et al. 2021). 7-Methoxycoumarin, which could be used as an organic synthesis intermediate, was also a drug with the effect of flat asthma, expectorant, cough suppression(Han et al. 2021).The basic function of melatonin was to participate in the antioxidant system and prevent cells from causing oxidative damage ,which was the strongest endogenous free radical scavenger ever found. In this respect, it outperforms all known substances in vivo(Cruz et al. 2014). Recent research proves that melatonin was the commander in chief of endocrine and it controlled the activity of various endocrine glands in the body, thus to indirectly control the function of our whole body(Cezary et al. 2021). The above analysis results revealed the differential metabolites between *Morchella* fruit bodies and mycelium that serving for the development and utilization of *Morchella* resources.

The enrichment analysis of different metabolites showed that different metabolites mainly participated in the synthesis of tyrosine, biosynthesis of thyroid hormones, synthesis of phenylalanine metabolites synthesis, linoleic acid metabolites synthesis, glycerol phosphate metabolites synthesis, metabolic pathway of choline in tumors, methyl butyl metabolites synthesis, arginine synthesis pathway, biosynthesis of arginine and proline metabolites(Dobson et al. 2012) that were the main signal ways of *Morchella* metabolism. The ways directly affected the accumulation of the key substances. The phenylalanine and tyrosine metabolic pathways mainly determined phenolic difference metabolites. The changes of these material content were related to the accumulation of phenylalanine and tyrosine, and indirectly affected the umami flavor of *Morchella*. Thyroid hormone signaling pathway was associated with improving the efficacy of myocardial ischemia and provided a theoretical basis for its clinical application(Zeng et al. 2019). Therefore, it was speculated that the active components in *Morchella* may achieve the purpose of improving myocardial ischemia through the key factors acting in the above signaling pathway. It provided a basis for the drug research of *Morchella* to improve myocardial ischemia,

and provided new ideas and methods for the research and development of traditional Chinese medicine. The biosynthesis pathways of arginine and proline metabolites could be a significant increase in anti-4-hydroxyl-L-proline and L-amphetamine-L-proline in the mycelium(He et al. 2019).

These findings have improved to our understanding of the molecular mechanisms accounting for the biological activities of *Morchella* fruit bodies and mycelium. It provides a basis for the drug research of *Morchella* to provide novel insight into further applications in drug industry.

## Conclusions

In this paper, the primary metabolites in fruit bodies and mycelium of *Morchella* based on extensive target metabolomics were analyzed. 354 significantly different metabolites were identified in the study. The first 9 of which can be clarified. The relationship between the detected various compounds and the pharmacological activity of *Morchella* may be as an orientation of scientific research for the development and utilization of *Morchella* in the future. Metabolic group information can provide references for the separation and purification identification of many active components in *Morchella*. Associated metabolic pathways also provide a basis for subsequent studies of the function of key genes in the metabolic pathways and the biosynthesis of the major metabolites.

## Declarations

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### Author contribution

Conceptualization, YY and ZW; software, JY; formal analysis, HW; investigation, YJ; resources,RJ; data curation, JL; writing—original draft preparation, YY;

writing—review and editing, YY; visualization, ZK; supervision, ZW.

All authors have read and agreed to the published version of the manuscript.

### Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

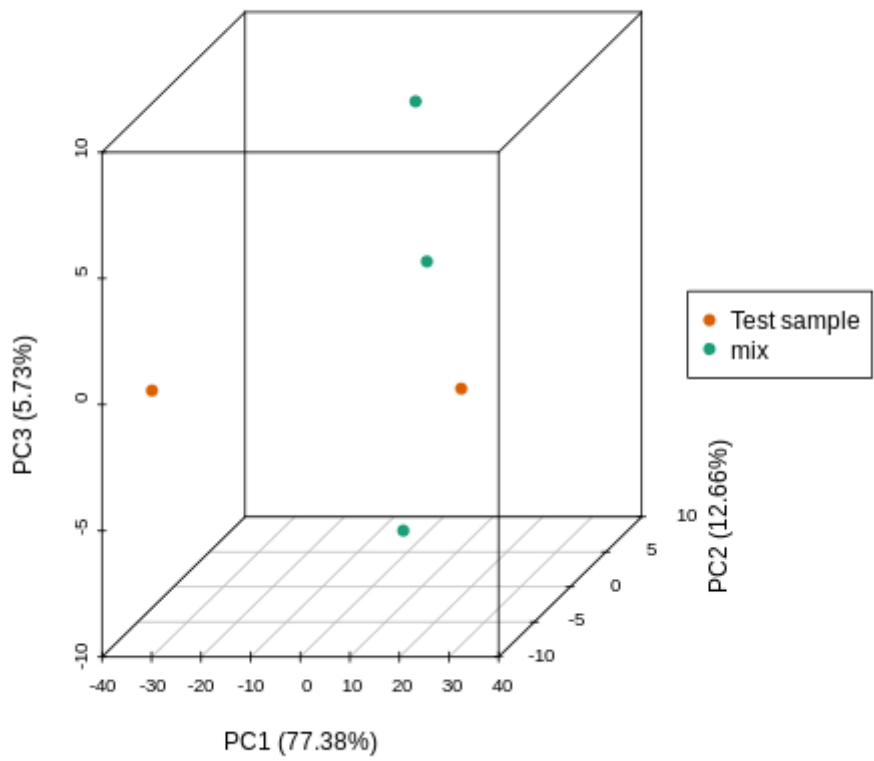
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## Figures



**Figure 1**

PCA score chart of the total sample

Scores OPLS-DA Plot

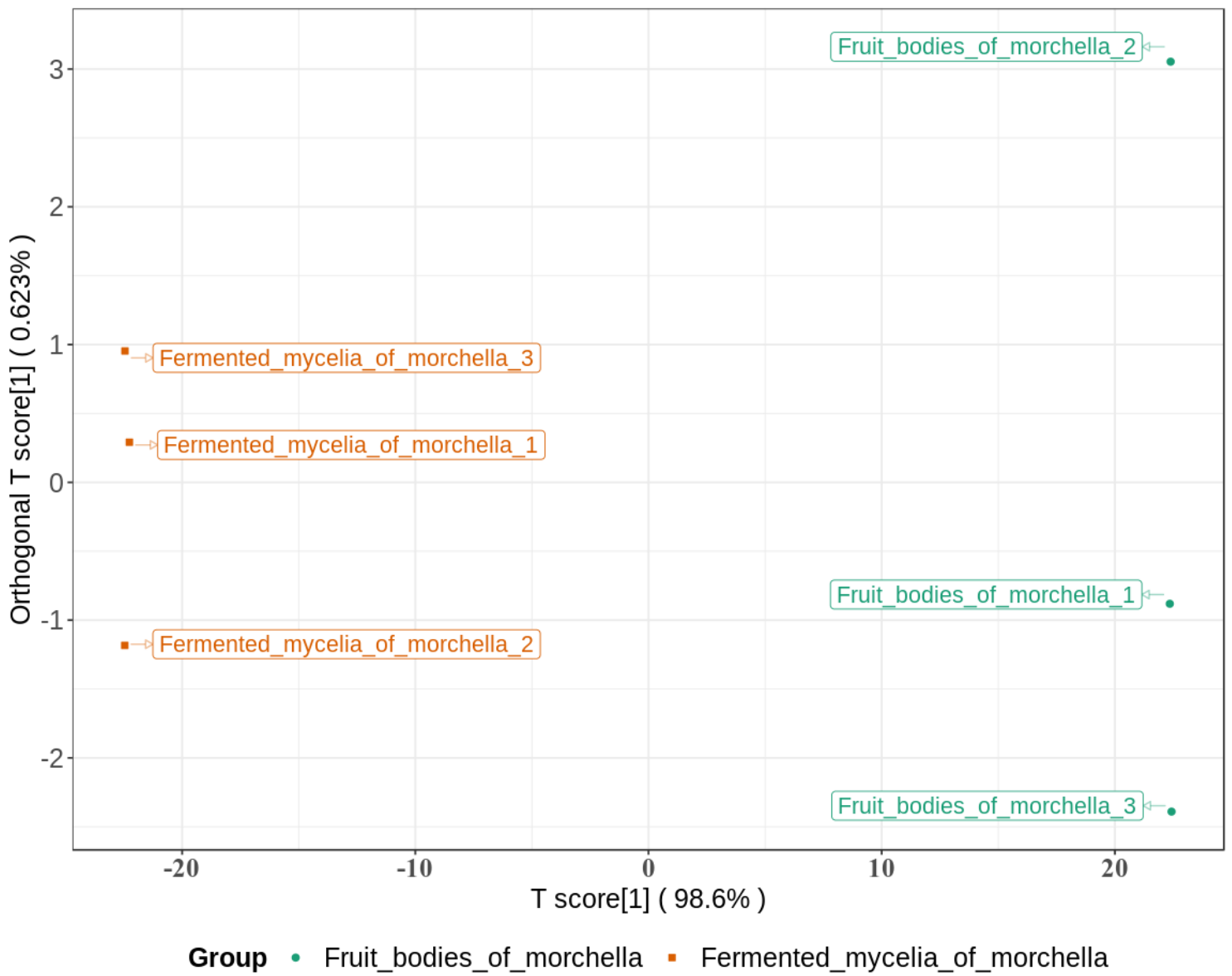


Figure 2

OPLS-DA score chart

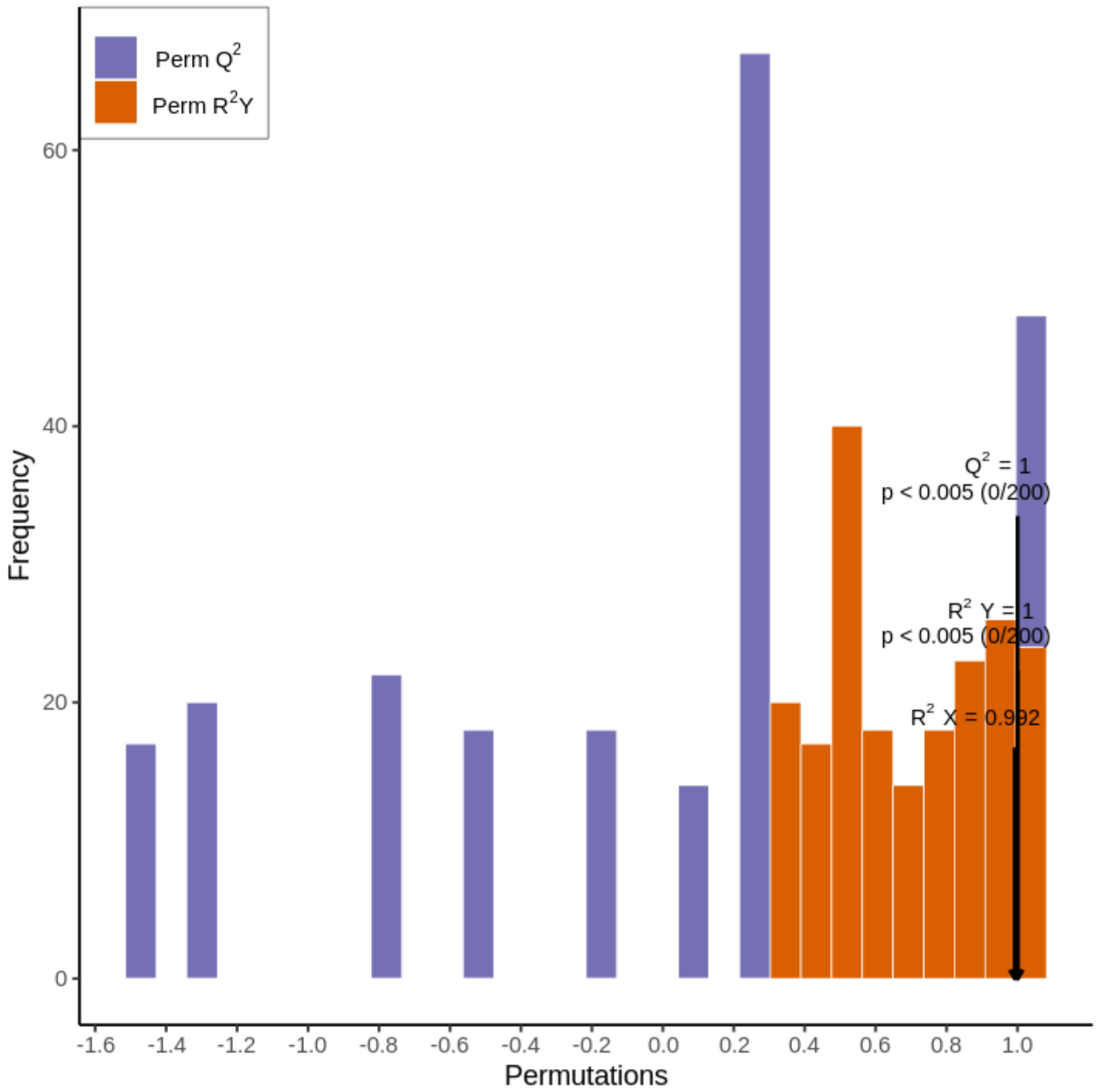
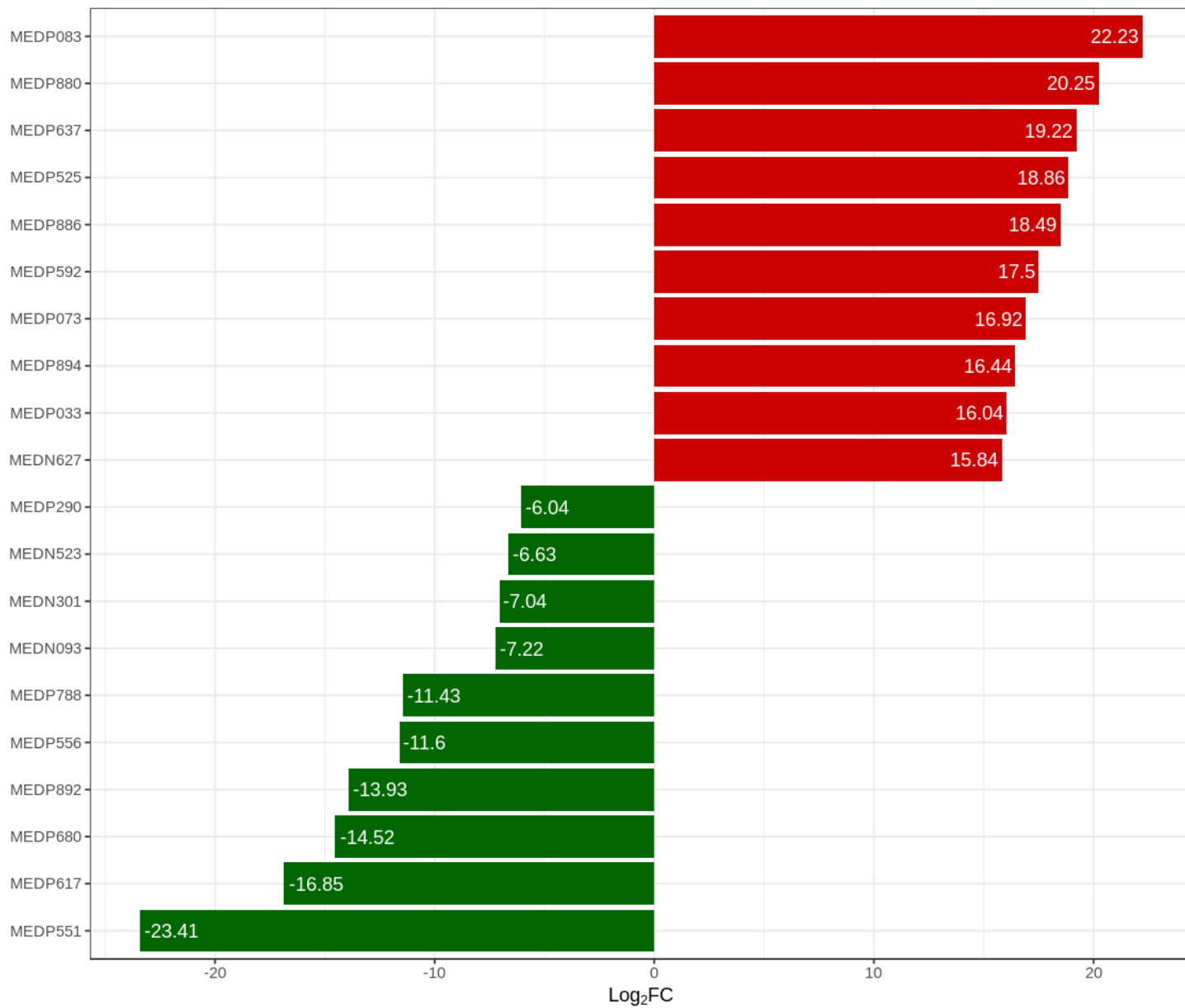


Figure 3

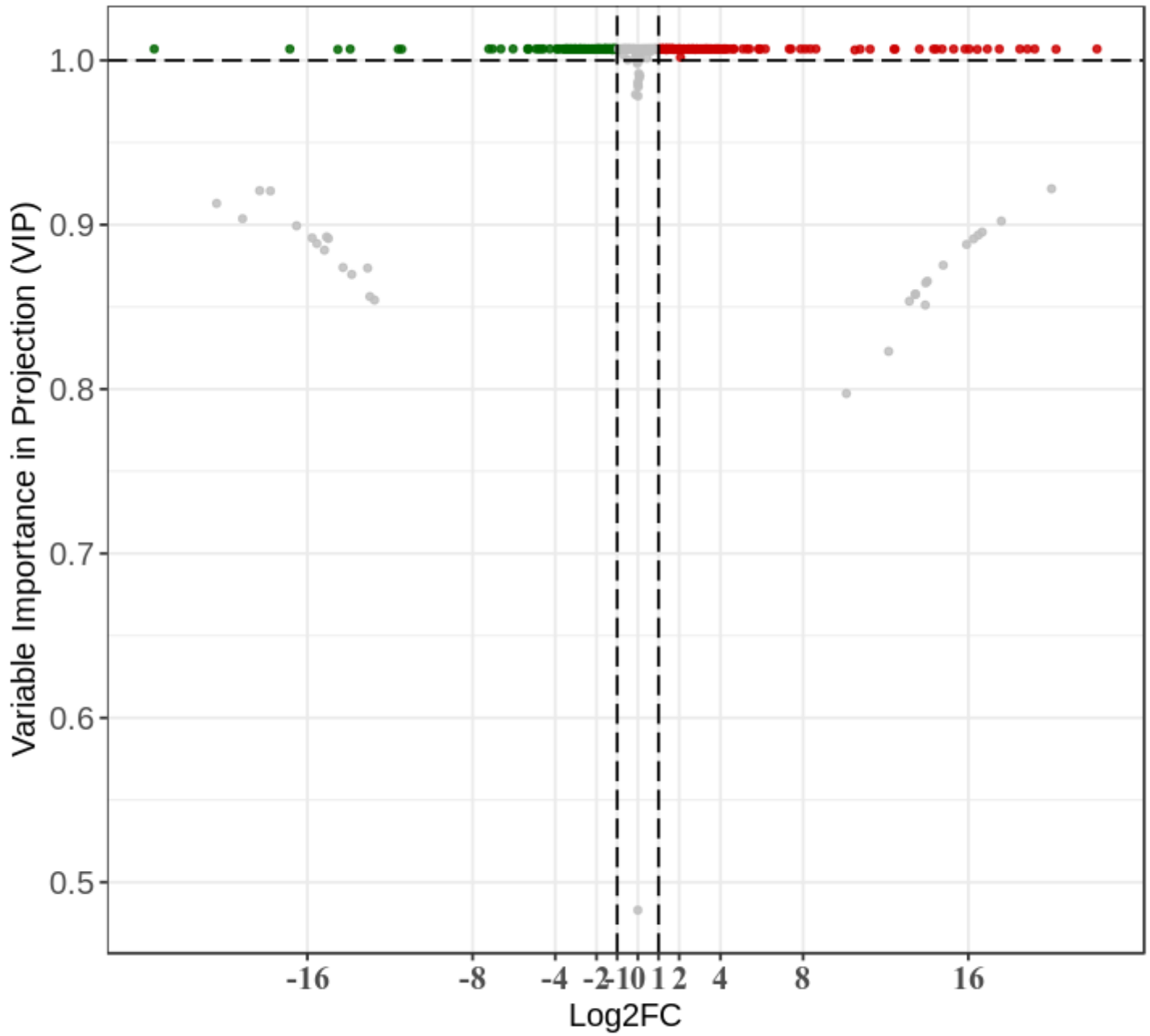
OPLS-DA verification diagram





**Figure 4**

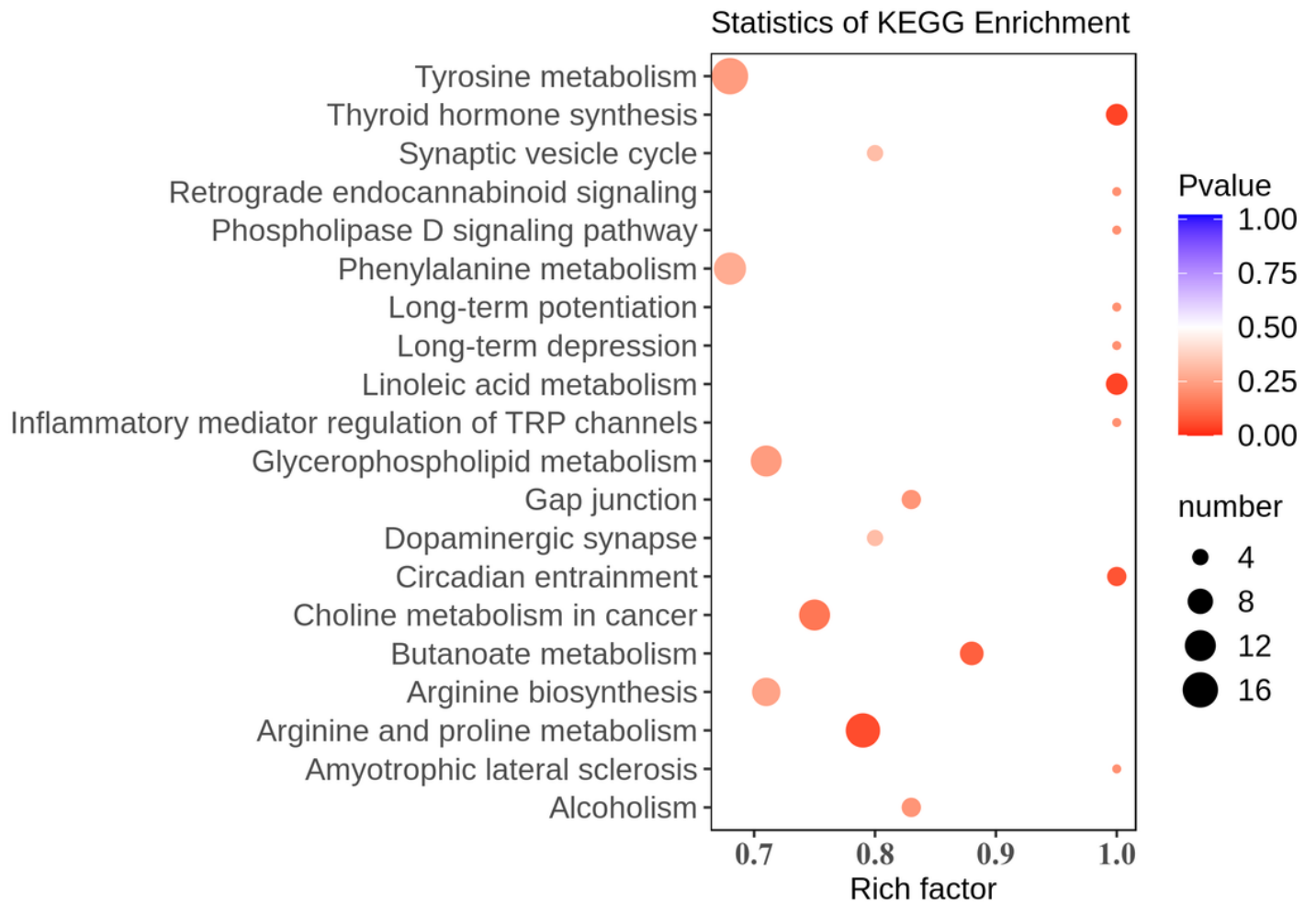
The 20 metabolites with the largest differences in content between the two samples Note: The top 10 metabolic components with the largest (red) and smallest (green) multiples of difference between morel fruit body and mycelium . MEDP083 : trans- 4 -hydroxy -L- proline; MEDP083 : N- α - acetyl- L- asparagine; MEDP033 : 3- iodotyrosine, MEDP083 : L- phenylpropan -L- proline acid ; MEDP525 : N- acetyl- -L- alanine; MEDP886 : N- amidino- L- aspartic acid; MEDP592 : 3- keto - sphingosine; MEDP073 : phenylacetyl glycine;MEDP894 : 3 -methoxytyramine; MEDN627 : ellagic acid; MEDP290 : sinapinic acid ; MEDN301 ; caffeic acid; MEDN523 : 3 -indole - lactic acid ; MEDN093 : 3- hydroxy -2- aminobenzoic acid ; MEDP788 : two Aniline; MEDP556 : 7 -methoxycoumarin; MEDP551 : N -methylephedrine; MEDP892 : Catechol; MEDP680 : hexylamine; MEDP617 : N-(2- furoyl ) glycine



**Statistics** • Down: 166 • Insignificant: 256 • Up: 188

**Figure 5**

Volcanic maps of differential metabolites Note: Green dots represent downregulated differentially expressed metabolites; red spots represented upregulated differentially expressed metabolites; and black represented non-differentially expressed metabolites.



**Figure 6**

Differential metabolite KEGG enrichment map Note: The abscissa represents the rich factor corresponding to each channel , the ordinate is the channel name, and the color of the point is pvalue . The more red, the more significant the enrichment. The size of the dot represents the number of different metabolites enriched.