

Treatment with Trace Amines Ameliorates Metabolic Dysfunction and Hair Thinning in High-Fat Diet-Induced Obese Mice

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General Introduction

The global prevalence of overweight and obesity has more than doubled since 1980 (Han et al., 2023). According to a 2009 report by the World Health Organization (WHO), over half a billion adults worldwide were overweight, and it is estimated that by 2030, 51% of the global population will be obese (Consultation, 2000; Wallace et al., 2022). Obesity is a significant risk factor for various diseases, including type 2 diabetes (T2D), cardiovascular disease, neoplasms, and dyslipidemia (Prasad et al., 2022). In parallel, non-alcoholic fatty liver disease (NAFLD), characterized by excessive lipid storage in hepatocytes, is a common hepatic manifestation of obesity-related metabolic dysfunction. NAFLD is often bidirectionally associated with obesity-related metabolic syndrome, increasing the risks of and contributing to the pathophysiology of atherosclerosis, cardiovascular diseases, diabetes, and chronic kidney disease (Im et al., 2020; Munkong et al., 2023; Suk et al., 2024). Recent research by Morinaga et al. (2021) has shown that stress induced by a high-fat diet (HFD) targets hair follicle stem cells (HFSCs), accelerating hair thinning in obese mice. Individuals with obesity frequently experience metabolic comorbidities that exacerbate disease-related consequences, posing significant public health challenges and resulting in substantial healthcare costs. Thus, there is an urgent need to prevent or control obesity. However, several approved obesity treatments and anti-obesity agents often carry side effects, including increased blood pressure, anxiety, and dizziness, and limitations related to potential drug abuse (Fabbrini et al., 2010).

Historically, natural products derived from animals, plants, or microbial fermentation have been used as medicines and have proven to be successful sources of potential drugs (Zhu et al., 2022). Therefore, a great deal of effort is being made to search for compounds that solve these problems from natural products that are consumed such as foods.

Trace amines (TAs), found within the central nervous system, include tryptamine, tyramine, octopamine, and phenylethylamine. These endogenous amine compounds are synthesized from precursor amino acids via aromatic-L-amino acid decarboxylase

(AADC) (Pei et al., 2016). As their name suggests, TAs are present at low levels within the mammalian/vertebrate brain, approximately 100 times lower than traditional monoamines such as dopamine, serotonin, or norepinephrine, with concentrations in the order of 0.1–10 nM (Zucchi et al., 2009). TAs are also produced in bacteria, fungi, and plant cells and can be found in certain foods, notably chocolate, aged cheese, and red wine (Branchek et al., 2003; Zheng et al., 2020). Traditionally, the functional effects of TAs have been described as neuromodulation, meaning they can either increase or decrease a cell's response to different neurotransmitters (Miller et al., 2011).

The anti-obesity effects of TAs have also garnered substantial scientific interest in recent years. According to Brial et al., mice with NAFLD chronically treated with octopamine exhibited reductions in liver histopathology and metabolic benefits (Brial et al., 2019). Additionally, Lee et al. (2022) reported that liver weight and the levels of alanine aminotransferase and total cholesterol were lower in obese mice induced by a high-fat diet (HFD) than in controls after treatment with a phytochemical combination containing octopamine, synephrine, and hispidulin. The previous study found that phenylethylamine, a dehydroxylated octopamine, identified in the water extract of chlorella (WEC) was beneficial to liver damage by decreasing the generation of methylglyoxal via increasing glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and consequently suppressing lipid peroxidation in the liver of obese mice chronically fed a HFD (Zheng et al., 2021). Furthermore, Kovacevic et al. (2019) formulated a novel shampoo containing tyramine, which reduced hair shedding by contracting the arrector pili muscle through the trace amine-associated receptor (TAAR). However, whether TA treatment can ameliorate HFD-induced hair thinning has not yet been studied. Based on these previous reports, it can be assumed that TAs may be useful in the prevention and treatment of obesity-related liver damage and hair thinning.

Accordingly, in Chapter 1, further investigation was conducted to study the molecular mechanisms underlying the action of phenylethylamine on (HFD)-induced obesity in mice using a targeted metabolomic approach based on high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS).

Additionally, changes in hepatic protein expression were detected using direct detection by anion exchange HPLC combined with SDS-PAGE and immunoblotting analyses. In Chapter 2, the presence of some TAs was examined, including tyramine, phenylethylamine, and tryptamine, in Hong Yao sour soup, a type of white sour soup traditionally used as a hair treatment in China. Furthermore, in Chapter 3, the interventional effect of TAs was evaluated by inducing a hair-thinning mouse model with a (HFD) and investigate the pigmentation activity of TAs on hair color in obese mice. These study results may provide a theoretical basis for preventing obesity caused by a (HFD) and offer insights into the high-value utilization of TAs.

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Chapter 1 Metabolomic and proteomic analysis of effects of chlorella water extract and phenylethylamine on high-fat diet-induced mouse liver damage

Abstract:

Recent studies have revealed that trace amounts of phenylethylamine (PHA) identified in water extract of chlorella (WEC) alleviated high-fat diet (HFD)-induced liver damage. To further investigate molecular mechanisms underlying the action of PHA, metabolomic and proteomic analyses were performed on liver of mouse from four groups: the normal diet-fed (ND), high-fat diet-fed (HFD), HFD mice supplemented with WEC (HFD-WEC) and supplemented with PHA (HFD-PHA) groups. Metabolites were evaluated by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Proteins were separated using anion exchange chromatography followed by SDS-PAGE. Differentially expressed proteins were identified by in the gel trypsin digestion followed by LC-MS/MS analysis or immunoblotting analysis. By utilizing MetaboAnalyst, principal component analysis (PCA) clearly distinguished ND and HFD groups, while no significant difference was observed between HFD-WEC and HFD-PHA. Twenty-four metabolic pathways in HFD-WEC and fifteen in HFD-PHA were significantly changed compared with HFD. Supplementation of WEC and PHA similarly changed protein expression compared to HFD group. Joint metabolomic and proteomic analyses revealed WEC and PHA had significant effects on the HFD mice livers via promoting the glucose metabolism, citrate cycle (TCA cycle), pyrimidine metabolism, arginine biosynthesis, and glutathione metabolism pathway. In conclusion, PHA in WEC is responsible for changes of the metabolites and critical enzymes in the liver by WEC.

1. Introduction

Currently overweight and obesity prevalence has steadily increased; it is one of the major public health problems that result in enormous costs to health-care systems. In United States where 42.4 % of adults classified as obese; by 2030, 51% of the global population are estimated to be obese (Kopelman et al., 2000; Wallace et al., 2022). Obesity refers to the accumulation of excessive body fat due to an imbalance between energy intake and energy expenditure, resulting in body mass index (BMI) value more than 30 (Consultation 2000). Considered as an increased oxidative status and a chronic inflammatory process, obesity is an epidemic threatening human wellbeing rather than a cosmetic problem, and particularly, is strongly associated with type 2 diabetes (T2D), cardiovascular disease, neoplasms, osteoarthritis, sleep apnea, and nonalcoholic fatty liver disease (NAFLD) (Guo et al., 2021). Currently, lifestyle intervention, pharmacotherapy, and bariatric surgery are main treatment options for the prevention or management of obesity and its complications (Carrasco-Pozo et al., 2019). However, the effectiveness of lifestyle intervention is usually and largely compromised due to low adherence. Pharmacotherapy is seriously challenged by the inevitable side effects and lose effectiveness in long-term treatments. In addition, bariatric surgery is invasive and expensive.

Consequently, the search for natural bioactives that minimize these undesirable side effects and are more tolerated in the prevention or management of obesity and regulating the disturbances observed in it, has been receiving increasing attention from the scientific community in recent years. In this sense, chlorella, a freshwater unicellular green alga, that is rich in nutrients and is commonly used as functional food and nutraceutical, has emerged as an alternative prophylactic treatment for obesity-related complications. Many clinical and experimental studies have shown that different species of chlorella, i.e., *C. pyrenoidosa* (CP) and *C. vulgaris* (CV), have a potential for alleviation of lifestyle-related symptoms, such as hypercholesteremia, hyperlipidemia, hyperglycemia, insulin resistance, and hypertension (Chu et al., 2022; Moradi et al.,

2021; Noguchi et al., 2016; Wan et al., 2018). In this regard, the previous studies have shown that water extract of CP (WEC) and PHA identified in it were beneficial to NAFLD by decreasing the generation of methylglyoxal via increasing glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and consequently suppressing the peroxidation of lipids in liver of obese mice after chronic being fed with high-fat diet (HFD). However, a comprehensive and complete view of metabolome and proteome that are changed in liver by supplementation with WEC and PHA to HFD mice is lacking.

Metabolomics analysis proposed by Nicholson et al. (1999) is defined as a comprehensive analysis, involving the identification and quantification of as more as possible metabolites that have low molecular weight (typically < 1500Da) in an organism, tissues, or cells. Currently, this tool plays a crucial role in the discovery of potential metabolic biomarkers in disease staging or diagnosis, as well for the monitoring of therapeutic intervention and response, including nutritional or functional food approaches. Proteomic analysis is a powerful tool in studying the changes in protein expression. In particular, the combined metabolomic and proteomic analyses can map significantly changed proteins together with significantly changed metabolites to metabolic pathways for functional enrichment analysis and pathway topology analysis; and jointly clarify the overall state of the biological status. The combined application of proteomic and metabolomic technology has undeniable advantages.

Objective of this study was to investigate the pathway alterations and molecular mechanisms underlying hepatoprotective effects by intervention of WEC and PHA to HFD-induced obese mice. For this purpose, a targeted metabolomic approach, based on high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) was applied. Moreover, changes in hepatic protein expression were detected using direct detection by anion exchange HPLC combined with SDS-PAGE and immunoblotting analyses. The results of the study may provide a theoretical basis for the prevention of obesity caused by HFD and the search for new healthy dietary patterns.

2. Materials and methods

2.1. Materials and reagents

Water extract of chlorella (WEC) was prepared and supplied by Sun Chlorella Co., Ltd. (Kyoto, Japan). Tween 20 was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Iodoacetamide (IAA) and glutaraldehyde were obtained from Wako Pure Chemical Industry (Osaka, Japan). 3-Nitrophenylhydrazine hydrochloride (3-NPH • HCl) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Coomassie brilliant blue R-250, dithiothreitol (DTT), sodium deoxycholate, silver nitrate, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), proteinase inhibitor cocktail, phenylethylamine, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC • HCl) and acetonitrile (HPLC grade) were purchased from Nacalai Tesque (Kyoto, Japan). Ammonium bicarbonate was purchased from Sigma Aldrich (St. Louis, MO, USA). Other chemicals used in this study were of analytical grade. Water purified through the Milli-Q system (Merck Millipore, Burlington, MA, USA) was employed.

2.2. Animals and diets

Liver, which were collected from mice (C57BL/6J) in the previous experiment (Zheng et al., 2021) and stored at -80 °C, were used. In the previous study, mice were randomly divided into the following four groups: ND group (n = 5) fed on a standard diet; HFD group (n = 5) fed on HFD (high-fat diet 32, CLEA Japan, Tokyo, Japan) comprises 32% crude fat with 60% of the calories derived from the fat; HFD-WEC group (n = 5) fed on HFD and orally administered with WEC (100 mg/kg bodyweight) through drinking water; HFD-PHA group (n = 4) fed on HFD and orally administered with PHA (10 µg/kg bodyweight) through drinking water.

2.3. Metabolomic data acquisition, processing, and statistical analyses

Aliquots of the frozen sample (approximately 100 mg) was mixed with 500 μ L of internal standard solution (10 μ M methionine sulfone) and homogenized in a BioMasher II (Nippi, Tokyo, Japan). The homogenates were mixed with 250 μ L of distilled water. The suspension (600 μ L) was mixed with 400 μ L of chloroform and vigorously agitated. After centrifugation at 9,100 g for 15 min at 4 °C, water layer (400 μ L) was collected and transferred to a centrifugal filter device (Amicon® Ultra Centrifugal Filter, 10 kDa MWCO). Filtration was performed at 9,100 g for 90 min at 4 °C. The filtrate was freeze dried and stored at -80 °C. The residue was dissolved with 50 μ L of distilled water and subjected to LC-MS/MS analyses.

The targeted metabolic profiling was carried out by Shimadzu Co., Ltd. using a Shimadzu Nexera X3 HPLC system (Shimadzu, Kyoto, Japan) coupled with an LCMS-8060NX triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Shimadzu). To quantify hydrophilic and hydrophobic metabolites, 2 different chromatographic separation methods were used. For hydrophobic compounds, reversed phase liquid chromatography separation was performed using a Shim-pack GIST PFPP column (2.1 mm $\times$ 150 mm, 3.0 μ m; Shimadzu). The binary gradient elution using mobile phase A (0.1 % formic acid in water) and mobile phase B (0.1 % formic acid in acetonitrile) at a flow rate of 0.25 mL/min was carried out. Column temperature was kept at 40°C. The gradient program was as follows: 0–2 min, 0 % B; 2–5 min, 0–25 % B; 5–11 min, 25–35 % B; 11–12.5 min 35–50%B; 12.5–16 min, 50 % B; 16–16.1 min, 50–95 %B;16.1–19 min, 95 % B; 19–19.1 min, 95–0 %B; 19.1–30 min, 0 % B. For hydrophilic and negatively charged compounds, ion-pair reversed phase liquid chromatography separation was performed using a Mastro2™ C18 (2.0 mm $\times$ 150 mm, 3.0 μ m; Shimadzu). The binary gradient elution used mobile phase A (10 mM dipentylamine and 15 mM acetic acid in water) and mobile phase B (methanol) at a flow rate of 0.3 mL/min was carried out. Column temperature was kept at 40°C. The gradient program was as follows: 0–0.5 min, 0 % B; 0.5–8 min, 0–15 % B; 8.0–12 min, 15–98 % B; 12–15 min, 98 % B; 15–15.1 min, 98–0 % B; 15.1–20 min, 0 % B.

Metabolites of total 135 compounds such as amino acids, carboxylic acids, carbohydrates, nucleic acids, nucleotides, and biogenic amines and etc., were identified and quantitated in the multiple reaction monitoring (MRM) mode operating under positive and negative ionizations. Detection parameters for each precursor/product ions of interest were obtained from the LC-MS/MS method package (Primary Metabolites Ver. 3, Shimadzu).

The generated LC-MS/MS data, peak intensity of compounds from livers were converted to a CSV format data matrix file using Microsoft Excel and uploaded to the MetaboAnalyst Ver. 5.0 web server (<https://www.metaboanalyst.ca/>) for statistical analyses. Before being analyzed, the data were normalized using range scaling option to achieve the normal distribution. Comparisons between groups for one component were performed using *t*-test and fold change (FC) analyses. Comparisons of all the four groups regarding multiple components were performed using principal component analysis (PCA) and partial least squares-discrimination analysis (PLS-DA). Comparisons between the two groups regarding multiple components were performed using orthogonal partial least squares-discrimination analysis (OPLS-DA). The quality of the OPLS-DA models was evaluated based on the R²Y (goodness-of-fit) and Q² (goodness-of-prediction) values. The metabolites reaching the threshold of $p < 0.05$ from the *t*-test and variable importance in the projection (VIP) >1 in the OPLS-DA were considered as significantly changed metabolites. Heatmaps were used for hierarchical clustering the samples.

The peak areas of significantly changed metabolites detected by OPLS-DA analysis were imported into MetaboAnalyst again for pathway analysis. To make the data normal distribution, peak areas were processed by median normalization, cube root transformation, and range scaling modes. Small Molecule Pathway Database (SMPDB) of mouse (*Mus musculus*) pathway library was selected. The pathway impact value of the metabolic pathways was calculated from pathway topology analysis and the *p* value of the metabolic pathways was calculated from the enrichment analysis. When the impact value was > 0.2 and the *p* value was < 0.05, these pathways were considered

significantly altered.

2.4. Hepatic proteomics analyses

Protein extraction from mouse liver

The frozen liver samples (approximately 50 mg) were homogenized in 15 volumes of 20 mM Tris-HCl buffer (pH 7.5), using a BioMasher II, then vortexed for 20 min at 4 °C. The homogenate was centrifuged at 12,000 g for 15 min at 4 °C. The supernatant from each was collected, pooled, and clarified through a 0.45- μ m filter. The protein concentration of the pooled sample was determined by a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) according to manufacturer's instruction.

Anion-exchange chromatography and Fractionation

A Shimadzu LC-10A HPLC system equipped with a Cosmogel IEX packed TypeQ column (50 mm $\times$ 4.6 mm, 1000Å; Nacalai Tesque) was used to fractionate the liver proteome. Mobile phase A was composed of 20 mM Tris-HCl buffer (pH 8.0), whereas mobile phase B was composed of the Tris-HCl buffer containing 0.5 M NaCl. The gradient program was as follows: 0-3 min, 0-15% B; 3-5 min, 15-35% B; 5-13 min, 35-75% B; 13-17 min, 75-100% B; 17-37 min, 100% B. The column was then equilibrated with at least 5 column volumes of mobile phase A before the next injection. Injection volume was 200 μ L with same protein content of each group. The flow rate was maintained at 0.5 mL/min. Fractions were collected every minute for 24 min, while the chromatogram was monitored through a UV Detector at 280 nm. All chromatographic runs were performed at room temperature.

Gel electrophoresis

One D SDS-PAGE was carried out on e-PAGEL HR 10% polyacrylamide precast gels (size: 90 mm with $\times$ 83 mm long $\times$ 1 mm thick; ATTO, Tokyo, Japan) by using the AE-6500 electrophoresis system (ATTO) filled with running buffer (0.025 M Tris-HCl, pH 8.3, 0.192 M glycine and 0.1% SDS). Aliquots of the fractions collected from the anion-exchange chromatography were mixed with a 5 times concentrated non-reducing

sample buffer (containing 0.25 M Tris-HCl, pH6.8, 0.25% Bromophenol blue, 50% Glycerol and 10% SDS) and denatured by heating at 98 ° C for 5 min. Aliquot of 24 µL was applied per well. Protein fractions were loaded side by side (i.e., ND fraction 1 next to HD fraction 1, then CD fraction 1, and at last the PHA fraction 1, etc.). Thus, protein bands in the same HPLC fraction from 4 groups could be compared on the same gel. Electrophoresis was run at 120 V constant voltage until the tracking dye reached the bottom of the gel.

Protein staining and proteomic data analysis

A combination of Coomassie brilliant blue R-250 (CBB) staining and silver staining were used to visualize the proteins. After electrophoresis, proteins in the gel were fixed in 10% (v/v) acetic acid and 40% (v/v) ethanol and stained overnight with staining solution containing 0.1% (w/v) CBB, 10% (v/v) acetic acid, and 45% (v/v) ethanol. Gels were destained in 10% (v/v) acetic acid and 40% (v/v) ethanol until the background became clear. After incorporation of the gel image at 600-dpi resolution with an Epson GT-X830 scanner (Epson, Nagano, Japan), silver staining was performed according to the methods of Oakley and Butcher. Briefly, the CBB stained gel were completely destained and fixed in a 10% (v/v) acetic acid and 40% (v/v) ethanol solution until the gel became colorless then washed with millQ water for 30 min. Sensitization of the gel was performed using 10% glutaraldehyde for 30 min, followed by six washes with millQ water. Thereafter, the gels were soaked in ammoniacal silver nitrate solution [0.076% (w/v) sodium hydroxide, 0.4%(v/v) ammonia, 0.8% (w/v) silver nitrate] for 15 min and washed with three changes of MillQ water for 5 min each. Finally, the gels were developed with a solution containing 0.005% citric acid and 0.019% formaldehyde, until the desired color intensity was attained. Staining was stopped by rinsing the gels in 5% (v/v) acetic acid. The silver-stained gels were scanned again.

The gel images were saved as 24-bit TIFF files and incorporated to an ImageJ software (Version 1.53, National Institutes of Health, USA) and converted to 8-bit type. Afterwards, gel band quantitative densitometric analysis was performed. The ratio of the quantitative values of each band relative to HFD group was calculated and indicated

as the FC values.

In-Gel Digestion

To identify changed proteins resolved by SDS-PAGE, the concentrated samples were prepared. Collected fractions were desalted by ZebaTM spin desalting column (Thermo Scientific) and subsequently concentrated via vacuum concentrator and subjected to SDS-PAGE again. After electrophoresis and CBB staining as described above, the bands of interest were excised, cut into small pieces, and washed twice with a solution consisting of 50% (v/v) methanol and 5% (v/v) acetic acid. Subsequently, the gel pieces were dehydrated with acetonitrile (approximately 100 μ L) for 5 min; the liquid phase was then discarded, and the pieces were completely dry in a vacuum concentrator. Proteins in gel pieces were reduced with 10 mM DTT in 100 mM ammonium bicarbonate for 30 min and then alkylated with 100 mM IAA in 100 mM ammonium bicarbonate for another 30 min. After reduction and alkylation, the gel pieces were dehydrated by addition of acetonitrile, rehydrated in 100 mM ammonium bicarbonate, and dehydrated again by acetonitrile. The liquid phase was discarded, and the gel pieces were completely dried in a vacuum concentrator again. The dried gel pieces were then incubated with 50 μ L Pierce trypsin (Thermo Scientific) solution (0.01 mg/mL in 100 mM ammonium bicarbonate) at 37 °C. After overnight digestion, the liquid phase was collected and peptides in gel piece were collected following extractions with 50 μ L of 50% (v/v) acetonitrile and 5% (v/v) formic acid. All extractions were repeated 3 times. All liquid phases were combined, filtered through the 0.45 μ m filters and concentrated to approximately 20 μ L.

Identification of proteins with LC-MS/MS and Database Searching

The peptides in the tryptic digest were resolved by a Shimadzu LC-20A HPLC system coupled to an LCMS-8040 triple quadrupole mass spectrometer (Shimadzu) with ESI source. Ten microliters (10 μ L) of tryptic peptide extraction were injected and separated on an Inertsil OSD-3 column (2.1 mm $\times$ 250 mm, 5 μ m; GL Science, Tokyo, Japan) at a column temperature of 40°C. A gradient LC system was operated using 0.1% formic

acid in water (Eluent A) and 0.1% formic acid in 80% acetonitrile (v/v) (Eluent B) at a flow of 0.2 mL/min. The gradient elution conditions were as follows: 0-15 min; 0-30% B, 15-20 min; 30-50% B, 20-25 min; 50-100% B, 25-30 min; 100% B, 30.1-40 min; 0% B. The MS data were acquired in positive mode with full scan range from m/z 240 to 1200. Capillary voltage was set to 4.5 kV. Nitrogen drying gas and nebulizing gas flow rates were 15 L/min and 3 L/min, respectively. Collision-induced dissociation gas (Ar) pressure was 224 kPa. Heat block temperature and desolvation line (DL) temperatures were set to 400 °C and 250 °C, respectively.

Protein database searching was performed with the MS-Fit program (<https://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msfitstandard>).

Monoisotopic mass to charge ratio values of peptide (m/z) was used for the search query. The search parameters were as follows: database, SwissProt; taxonomy, *Mus Musculus*; digest, trypsin; mass tolerance, 0.6 Da; constant modifications, carbamidomethylation of cysteine; possible modifications, oxidation of methionine; charge state, 2. Each protein identification was based on a minimum of four peptide hits with high quality MOWSE scores (approximately $>1E+6$). The match peptides were then performed the sequence analysis using the same instrument and LC condition, by product ion scan at a series of collision energy.

Detection of differential proteins by immunoblotting

The frozen liver samples (approximately 50 mg) were homogenized with 300 μ L of RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA) containing 1% proteinase inhibitor using a BioMasher II, then vortexed for 20 min at 4 °C. The homogenate was centrifuged at 12,000 g for 15 min at 4 °C. The protein concentration in the supernatant was measured using a BCA protein assay kit and the concentration was adjusted to 10 μ g/15 μ L with the RIPA buffer. Then samples were mixed with the appropriate volume of 2 times concentrated Laemmli's sample buffer, boiled for 5 min at 98°C. Electrophoresis was performed as mentioned above. To monitor transfer efficiency, all samples were mixed with the same volume of pre-stained marker (Prestained XL-Ladder, Integrall, Tokushima, Japan) to ensure the

consistency of transfer efficiency. The resolved proteins were transferred from the gel to polyvinylidene difluoride (PVDF) membranes (0.45 μm ; GE Healthcare Life Sciences, Chicago, IL, USA) using a semi-dry blotting apparatus (WSE 4020, Atto, Tokyo, Japan). The band intensity of pre-stained markers was quantified using ImageJ 1.52a. The PVDF membranes were blocked with 4% Block Ace solution (Megmilk Snow Brand, Sapporo, Japan) at room temperature (25 °C) for 30 min and washed three times with 50 mM Tris-HCl buffer (pH 7.5) containing 2.68 mM KCl, 137 mM NaCl, and 0.05% (v/v) Tween 20 (TBST). Then, membranes were incubated overnight at 4 °C in the following primary antibodies with the respective dilutions: rabbit anti-glucokinase (GCK) (Sigma-Aldrich, SAB5700828, 1:1000), rabbit anti-phosphofructokinase, liver type (PFKL) (Sigma-Aldrich, AV45774, 1:1000), rabbit anti-argininosuccinate synthase (ASS) (Sigma-Aldrich, AV41366, 1:1000), rabbit anti-pyruvate dehydrogenase E1 subunit alpha 1 (PDHA1) (Sigma-Aldrich, AV48136, 1:1000), rabbit anti-pyruvic carboxylase (PC) (Sigma-Aldrich, SAB4502091, 1:1000), rabbit anti-glutathione S-transferase A3 (GSTA3) (Thermo Fisher, PA5-119147, 1:1000) and horseradish peroxidase (HRP) -conjugated secondary antibody (Cell Signaling Technology, 1:8000) for 1 h at room temperature, followed by washing with TBST (3 times). The membranes were covered with the Chemi-Lumi One Super chemiluminescence assay working solution (Nacalai Tesque) and incubated for 1 min. Immunoreactive bands were detected using a Lumino Graph I system (Atto). ImageJ software was used for gel band quantitative densitometric analysis.

The fold change (FC) of protein band intensity was calculated using average peak intensities in comparison of two groups (HFD group/HFD-WEC group and HFD group/HFD-PHA group).

2.5. Combined multi-omics analysis

FC values of metabolites and proteins were Log₂-transformed, and then the metabolites and proteins with a Log₂(FC) of more than 1 or less than -1 were selected. Furthermore, by entering the metabolites and proteins with Log₂(FC) values in the Joint-Pathway

Analysis module of MetaboAnalyst 5.0, the integrated pathways were determined. Enrichment analysis and topology analysis were performed using Hypergeometric Test and Degree Centrality, respectively.

2.6. Quantitation of central carbon metabolism carboxylic acids in liver

The carboxylic acids that are among central carbohydrate metabolism (CCM) pathway including glycolysis, gluconeogenesis, and the TCA cycle in liver. However, some carboxylic acids (key metabolites of carbohydrate) were not detected by the metabolomic analysis. Thus, these carboxylic acids were quantitated using a 3-NPH derivatization strategy. Briefly, individual stock solutions of each analyte (10 mM) were prepared by dissolving standards in water, including pyruvate, fumarate, and oxaloacetate. Mixed working solutions were prepared in the range of 0.01–100 μ M by diluting adequate amounts of each individual stock solution in methanol / water (50:50, v/v). A 100 mg liver samples were weighed and homogenized with 1.0 mL of methanol/water solution (50:50, v/v) using a BioMasher II. Supernatants were collected after centrifugation performed at 16000 g for 10 min at 4°C and dried in a vacuum concentrator. The dry extracts were reconstituted in 200 μ L of acetonitrile/water (50:50, v/v). For derivatization, aliquots (100 μ L) of each mixed working solutions or the liver reconstituted samples were mixed with 50 μ L of 200 mM 3-NPH in methanol / water (50:50, v/v) and 50 μ L of 120 mM EDC in methanol / pyridine (94:6, v/v). Once mixed, the solutions were reacted in an ice water bath (0°C) for 60 min. After reaction, each solution was rapidly diluted with 800 μ L of acetonitrile / water (50:50, v/v). After being filtered through a 0.45 μ m filter, an aliquot (5 μ L) was injected into the same LC–MS/MS system (LCMS-8040). Chromatographic separation was achieved using a Cosmosil 5C18-MS-II column (150 mm $\times$ 2.0 mm, 5 μ m, Nacalai Tesque) maintained at 40 °C. A gradient of formic acid (0.1%) in water (solvent A) and formic acid (0.1%) in acetonitrile (solvent B) at a flow of 0.2 mL/min was used for separation. The gradient elution conditions were as follows: 0-1 min, 25% B; 1-13 min, 25-95% B; 13-18 min,

95% B; 18-18.1 min, 95-25% B; 18.1-24 min, 25% B. The MS acquisition methods used negative electrospray ionization (ESI) and analysis in multiple reaction monitoring (MRM) mode. Other LC-MS conditions remained unchanged.

2.7. Statistical analysis

Other statistical analyses were performed using GraphPad Prism (version 5.0; San Diego, CA, USA). Data were expressed as mean $\pm$ standard deviation (SD). Comparisons among multiple groups were evaluated using one-way ANOVA with Dunnett's multiple comparison test. Statistical significance was determined at $p < 0.05$.

3. Results

3.1. Metabolomic analysis

Based on 135 metabolites identified and quantified by LC-MS/MS (Supplemental Table 1), PCA and PLS-DA score plots were established to gain an overview of data and understand the similarities/dissimilarities in metabolite contents among different groups. As seen in the PCA score plot of all groups (Figure 1A), a clear separation of the 95% confidence ellipse regions between the ND and HFD groups was observed, indicating that the significant metabolic differences were induced by HFD feeding. The 95% confidence ellipse regions from mouse treated with WEC and PHA mostly overlapped, indicating a high similarity among the two groups. These facts indicate that PHA is responsible for the modulation of metabolites induced by WEC, while its content in WEC is very low (12.5 $\mu\text{g/g}$) (Zheng et al., 2021).

PCA is an unsupervised model and belongs to exploratory analysis. Due to the complexity of metabolomics data, PCA method may not fully distinguish the groups for samples with insufficient differences. In contrast, PLS-DA is a supervised classification method, which can provide better information of difference between groups and predict the grouping of samples. The PLS-DA score plot showed evident

separation between the four groups (Figure 1B). In addition, the ND group located on the opposite side of HFD group and the HFD-WEC and HFD-PHA groups showed moving towards the ND group in Component 1 direction; moreover, both HFD-WEC and HFD-PHA groups located closer to the ND group of mice indicating that the changes in metabolic profile caused by HFD might be partially restored by WEC and PHA intervention.

To better visualize the patterns of metabolites, heatmap and hierarchical clustering were made. Heatmap and dendrograms on rows showed distinct discrimination between ND and HFD groups (Supplemental Figure 1). Of interest, HFD-WEC and HFD-PHA groups clustered together. Also, according to hierarchical clustering performed on the compounds, these metabolites can be divided into four groups. Supplementation of WEC and PHA did not change metabolites in group I compared to HFD group. However, metabolites in groups II and IV changed by supplementation of WEC and PHA, resulting in a pattern similar to that of the ND group. The supplementation increased the metabolites in group III compared to both ND and HFD groups. These results are consistent with the views in the PCA and PLS-DA.

The OPLS-DA models were built to reveal clear discrimination of liver metabolites in WEC (Figure 1C) and PHA groups (Figure 1D) compared to HFD group. Then, the permutation test of the OPLS-DA was performed. For the comparison between HFD and HFD-WEC groups (Figure 1C), the parameters; R^2Y and Q^2 were 0.953 and $Q^2=0.904$, respectively. Similarly, R^2Y and Q^2 values were 0.899 and 0.840 for the comparison between HFD and HFD-PHA groups. These high values indicated that both analyses exhibited excellent fitness and predictive ability. Thus, supplementation of WEC and PHA distinctly changed metabolites in liver of mice fed HFD, whereas dose of PHA was very small (10 $\mu\text{g/kg}$ body weight).

To choose significantly changed metabolites in liver by supplementation of WEC and PHA to mice fed HFD, VIP score of metabolites were obtained from OPLS-DA, which reflects the contribution of the analyzed variables to the model. Figure 1 E and F show the top 15 metabolites that primarily contributed to the separation in OPLS-DA.

Finally, significantly changed metabolites of HFD-WEC and HFD-PHA were screened based on VIP scores >1.0 and $p < 0.05$ by comparing them with HFD groups (Supplemental Table 2 and Table 3). The heat map for two groups visually showed the differences of significantly changed metabolites by supplementation of WEC and PHA (Figure 2A and B). Supplementation of WEC and PHA significantly increased 48 and 34 metabolites and significantly decreased 19 and 32 metabolites, respectively.

Pathway analysis of these significantly changed metabolites indicates that 24 and 15 metabolic pathways significantly (p values < 0.05) changed with high impact values (> 0.2) (Supplemental Table 4 and 5). Figure 3A and B show the top 10 significantly enriched pathways by supplementation of WEC and PHA. Apparently among all these pathways, gluconeogenesis, glycolysis, glycerolipid metabolism, glutathione metabolism, arginine and proline metabolism, fructose and mannose degradation and proline metabolism, and cardiolipin biosynthesis were shared by both HFD/HFD-WEC and HFD/HFD-PHA groups.

3.2. Proteomics analysis

To determine the effect of WEC and PHA treatment on protein expression in HFD feeding mice, a proteomics platform was utilized, including protein separation technique via anion exchange chromatography followed by 1D-SDS-PAGE separation. The protein concentration of the liver lysates from four mouse groups was adjusted to 5 mg/mL. Aliquot (200 μ L) of liver lysates were sequentially separated on the column packed with strong anion-exchanger Type Q based on protein charge. Representative anion exchange chromatograms of the four groups are shown overlaid in Figure 4. Following of unbound proteins, bound proteins were eluted with three-steps gradient of NaCl. Chromatographic differences are observed among the four groups (Figure 4).

Twenty-four fractions were collected from each anion chromatography experiment. Same HPLC fractions from 4 groups were loaded side-by-side onto a 1D-SDS-PAGE for the second-dimension protein separation. The gels were visualized with CBB and silver stain for differential band analysis (Supplemental Figure 2A and 2B). The initial

impression after the second-dimension separation was that liver samples from the ND, HFD-WEC, and HFD-PHA group presented similar electrophoretic profiles, whereas liver sample from the HFD group presented a visible difference from other three groups. By densitometry analysis of 1D-SDS-PAGE, 11 bands shown in the boxes in Figure 5A were selected and the band intensities were compared. Relative log₂FC was calculated for gel band pairs based on the relative intensities among HFD vs HFD-WEC, and vs HFD-PHA groups (Figure 5B). As observed Figure 5B, 9 proteins were up-regulated and 2 proteins down-regulated in the two treatment groups compared with the HFD group. Except for protein band (Fr17A), all proteins changed to same direction by the two supplementations. These 11 protein bands were excised from the gel and subjected to in-gel digestion for protein identification. Table 1 summarizes the identified protein, band number, and the fold change. According to their functional properties, the identified proteins were grouped into the following four categories: Proteins involved in carbohydrate and energy metabolism, such as fructose-bisphosphate aldolase B, alpha-enolase, and aconitate hydratase; Proteins involved in amino acid metabolism, such as ornithine transcarbamylase and carbamoyl-phosphate synthase; Proteins involved in detoxification, such as glutathione S-transferases P1, and unknown functional proteins, such as the Annexin A5.

Based on the results of proteome and metabolome analyses, immunoblotting was carried out to verify the expression changes of 6 additional key proteins involved in glycolysis, gluconeogenesis, and arginine biosynthesis (Figure 6A). As shown in Figure 6B, log₂FC was calculated for protein band pairs based on the relative intensities among HFD vs HFD-WEC and vs HFD-PHA groups. Finally, combined both the results of HPLC-1-D-SDS-PAGE and immunoblotting, proteins with the threshold of log₂FC >1 or < -1 were considered as differentially expressed between HFD group and the treatment groups. A total of 6 proteins were differentially expressed in the HFD-WEC group compared with the HFD group including carbonic anhydrase 3, aconitate hydratase, alpha-enolase, argininosuccinate synthase (ASS), carbamoyl-phosphate synthase, and pyruvic carboxylase (PC). Similarly, 6 differentially expressed proteins

were also found in the HFD-PHA group compared with the HFD group including ASS, glutathione S-transferase P1, aconitate hydratase, alpha-enolase, carbamoyl-phosphate synthase, and PC.

3.3. Combined multi-omics analysis

The data of 6 differentially expressed proteins and 46 metabolites with $\log_2FC >1$ or < -1 were loaded to the MetaboAnalyst 5.0 to analyze the effect of the whole effect of WEC in the liver of mice fed HFD. As shown in Figure 7A, six pathways; purine metabolism, pyrimidine metabolism, citrate cycle (TCA cycle), glycolysis or gluconeogenesis, arginine biosynthesis, and pentose phosphate pathway were significantly changed. After PHA supplantation, 6 differentially expressed proteins and 46 metabolites with $\log_2FC >1$ or < -1 jointly reveal significant changes of 5 metabolic pathways: pentose phosphate pathway, pyrimidine metabolism, arginine biosynthesis, glycolysis or gluconeogenesis, citrate cycle (TCA cycle), and glutathione metabolism (Figure 7B). All pathways changed by supplementation with PHA (B) were shared by those by supplementation of WEC (B) (Supplemental Table 6 and Table 7).

3.4. Quantitation of central carbon metabolism carboxylic acids in liver

To enable a highly sensitive MS detection of CCM-related carboxylates characterized by low ionization efficiency and poor chromatographic retention on the reversed-phase LC column, samples of liver were derivatized with 3-NPH in the presence of EDC as a coupling reagent and then directly analyzed by means of LC-MS/MS. After optimization of the derivatization parameters, effective separations were achieved for the 3 derivatives. A highly sensitive MS detection over a large concentration range could be achieved and all standards exhibited good linearity ($R^2 > 0.99$), ensuring dependable quantification. Their concentrations in liver were calculated by referring to the external standard's calibration curve. As shown in Figure 8, the concentrations of

pyruvate and oxaloacetate were significantly decreased, while fumarate was significantly increased in HFD group compared with the ND group. WEC and PHA administration corrected pyruvate change induced by the consumption of an HFD. Moreover, WEC and PHA showed the effect on raising fumarate.

4. Discussion

The formation and development of NAFLD are complex, which are associated with lipid over-accumulation in the liver, inflammation, oxidative stress, gut microbial imbalance, etc. (Zhang et al., 2024). Day et al. (1998) proposed a “two-hit” hypothesis that NAFLD pathogenesis is initiated by the first producing steatosis and then promoted by oxidative stress capable of generation of liver damage. Unfortunately, the current therapeutic outcomes for dealing with NAFLD are unsatisfactory (Kumar et al., 2021). The previous study showed that supplementation of WEC and very low dose of PHA (10 µg/kg body weight) attenuated liver damage without decreasing fat accumulation in liver of mice fed HFD. Supplementation of WEC and PHA attenuated HFD-induced decrease of GAPDH in liver. These facts suggest that HFD negatively impacts on carbohydrate metabolism which can cause liver damage. However, effects of supplementation of WEC and PHA on the metabolism remained to be solved.

In the present study, firstly, a targeted metabolomics approach based on LC-MS/MS was performed to explore the characteristics of liver metabolism in mice of each group. The liver is crucial for the maintenance of normal glucose homeostasis; it produces glucose during fasting and metabolizes glucose postprandially. It has been reported that HFD feeding down regulated the glycolysis and TCA cycle and resulted in significant abnormal glucose metabolism in both mice and rats (Chao et al., 2014; Zhou et al., 2012; Zhu et al., 2020). Moreover, it is also reported that the HFD enhances lipid oxidation (Yang et al., 2020). In general, when sufficient concentrations of acetyl-CoA (the oxidation of fats) or citrate (the product of acetyl-CoA condensation with oxaloacetate) are present, gluconeogenesis is favored, which may lead to switch from

net hepatic glucose consumption to glucose output and aggravate hyperglycemia in mice consuming HFD. On the other hand, ectopic lipid accumulation in the liver proved to cause hepatic insulin resistance and plays a major role in the development of type 2 diabetes (T2D) (Samuel and Shulman 2012). Hepatic insulin resistance is characterized by the inability of insulin to stimulate hepatic glycogen synthesis and suppress gluconeogenesis under postprandial conditions, then lead to change net flux toward gluconeogenesis rather than glycolysis, worsening of hyperglycemia, and overt T2D (Goedeke, et al. 2019). Similar findings were observed in the present metabonomic results. It can be shown that the level of metabolites related to anaerobic (pyruvate acid) and aerobic (citric acid) were decreased after HFD diet feeding when compared with ND. Although the levels of glucose in serum were not measured in this study, the present study revealed that WEC and PHA administration raised glycolysis intermediates, such as phosphoenolpyruvate, 2-phosphoglycerate, 2-phosphoglycerate, and pyruvate acid. In addition, the previous studies have shown that WEC and PHA increased hepatic GAPDH, however the levels of the glucose 6-phosphate, fructose 1,6-bisphosphate, PFKL did not significantly increase by supplementation with WEC and PHA in the current study, and the levels of GCK slight increased. These results demonstrated that WEC and PHA treatment does seem to have an effect in NAFLD mice and lead to change net flux toward glycolysis, which occurs via an improvement in the payoff phase of glycolysis rather than preparatory phase.

Next, the quantitative protein expression profile among groups were analyzed. Consequently, the level of proteins involved in detoxification, energy metabolism, and amino acid metabolism were higher in the HFD-WEC and HFD-PHA group than in the HFD group. The current results showed that WEC and PHA administration increased the expression of the liver antioxidant enzyme-glutathione S-transferase (GST). GST is a glutathione sulfur transferase and essential for metabolizing prooxidant xenobiotics in the liver. There is some evidence that HFD can downregulate its expression in the liver. Aerobic organisms inevitably produce reactive oxygen species (ROS), which are byproducts of oxidative metabolism that could induce oxidative damage to cells. In

general, a cell has antioxidant enzymes that protect against the detrimental effects of ROS. However, disrupting the ROS-antioxidant balance under physiological conditions leads to excessive ROS production and the prevalence of metabolic diseases. In this chapter, data showed an increased expression of liver GST P1 after WEC and PHA administration, suggesting that WEC and PHA, even with HFD, can increase the levels of antioxidant enzymes in the liver.

Acetyl-CoA enters TCA cycle by condensing with oxaloacetate to form citrate. The citrate is then converted to isocitrate through the action of aconitate hydratase. Proteomic analysis showed the aconitate hydratase was down-regulated by HFD. Loss of aconitate hydratase activity limits TCA cycle activity, which will favor the translocation of citrate from mitochondria to cytosol to undergo fatty acid synthesis. Traditionally, pyruvate carboxylase (PC) catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetate, which is the first regulated step in the gluconeogenic pathway of pyruvate and its precursors. However, new evidence that PC plays an important role in TCA cycle has also been given. Cappel et al. (2019) report that, in the liver, PC maintains TCA cycle intermediates, and that its loss is insufficiently compensated by alternative pathways. Then they further emphasize a point that PC flux is essential for TCA-cycle-dependent biosynthesis. Loss of hepatic PC worsens liver inflammation in obesity. In the current study, decreased aconitate hydratase and PC levels were observed in HFD mice, WEC and PHA reversed these trends. Moreover, the administration of PHA increased liver citric acid level, while level of oxaloacetic acid did not significantly affect, which may be due to the increased PC flux. Considering the above results, it is speculated that WEC and PHA prevented and improved distorted glucose metabolism induced by the HFD via the glycolysis-TCA cycle.

The pentose phase pathway is a method of oxidative decomposition of glucose in tissues (liver, adipose tissue, etc.), which promotes the utilization of glucose, reduces blood glucose level, and produces a large amount of NADPH (reducing agent). There is an increase amount of evidence proving that HFD consumption contributes to the progression of increases lipid peroxidation. Peroxidation of membrane lipids may cause

cell necrosis and megamitochondria. The aldehyde products of lipid peroxidation, 4-hydroxynonenal and malondialdehyde (MDA) may also contribute to inflammation. The pentose phase pathway is one of the main antioxidant defenses. NADPH maintains the anti-oxidative power of glutathione, increases the antioxidant status in the cells, and then protects cells from damages caused by lipid peroxides, free radicals and peroxides. In the current experiment, a significant increase of NADPH in the PHA group compared to the HFD group was detected, indicating that PHA increased the glucose metabolism via pentose phosphate pathway to increase anti-oxidative capacity in liver of mice fed HFD. Increase of NADPH by administration of WEC and PHA can contribute to mitigation of the HFD-induced enhanced lipid peroxidation. As a result, the integrated pathways of glycolysis, TCA cycle, and pentose phase pathway with proteomic and metabolomic data indicated that supplementation of WEC and PHA enhanced not only energy expenditure of carbohydrates but also anti-oxidation in liver.

Additionally, the urea cycle is the major pathway for detoxification of ammonia and nitrogen excretion in mammals. Carbamoyl-phosphate synthetase 1 (CPS1) and ornithine transcarbamoylase (OTC) are mitochondrial enzymes catalyzing the first and second steps of the urea cycle, respectively. The expression of both enzymes is higher in hepatocytes than in other tissues. Deficiency and reduced activity of CPS1/OTC cause hyperammonaemia. Argininosuccinate synthetase (ASS) catalyzes the reversible condensation of citrulline with aspartate to form argininosuccinate. ASS is a highly conserved enzymes expressed in various tissues. Decreased expression of ASS attenuates the metabolism of ammonia and formation of argininosuccinate, resulting in abnormally higher concentration of ammonia and citrulline in the blood. It has been reported that within the urea cycle, the enzymes involved, such as CPS1, OTC, ASS, argininosuccinate lyase (ASL), and arginase 1 (ARG1), were downregulated by the HFD. Similar findings were observed in the proteomic result when compared the HFD group to ND group. The groups treated with WEC and PHA showed recovery patterns for these enzymes. From these results, it appears that the HFD induces alterations in the urea cycle, which can be ameliorated by supplementation of WEC and PHA.

The trace amines (TAs), including octopamine, synephrine, tryptamine, tyramine, and PHA, present at much lower concentrations in the central nervous system (CNS), and hence referred to as “trace” amines. TAs are now recognized as neuromodulators to play significant neurophysiological and behavioral functions. These compounds exert their effect by activating a class of G protein-coupled receptors termed Trace-Amine Associated Receptors (TAARs). Of the entire family of TAAR receptors, only TAAR1 and TAAR4 (previously TA1 and TA2) have been shown to be activated by TAs. TAAR9 mRNA and other TAARs were identified in the mucosal layer of the gastrointestinal tract (Zhukov et al. 2022).

Although, its molecular interactions and downstream targets have not been fully elucidated, it has been reported that TAAR1 activation triggers cellular signaling events including activation of PKA and PKC that lead to phosphorylation dependent dopamine efflux via the dopamine transporter and dopamine transporter internalization (Xie et al., 2007; Xie et al., 2008; Xie et al., 2009). In addition, TAAR1 agonists (such as amphetamine, methamphetamine or trace amine) compete with dopamine for reuptake (competitive inhibition) (Miller et al., 2011). These processes raise extracellular dopamine and norepinephrine levels and were documented to cause amphetamine-like effects. Benkirane et al. (1986) reported that exposure to amphetamine, phenylethylamine, m- or p-tyramine, increased in a concentration-dependent manner the spontaneous outflow of [³H]-noradrenaline from atrial slices. Baker et al. (1976) reported that phenylethylamine caused considerable increase in the release of ³H-dopamine from striatal synaptosomes (133 % increase during 5 min) and the release of ³H-noradrenaline (38 % increase) from hypothalamic synaptosomes. Goldstein et al. (1983) reported that injecting of d-amphetamine and tyramine (Tyr) dramatically increased systolic pressure (BPs) associated with increased plasma norepinephrine (NE) but unassociated with changes in plasma epinephrine or heart rate (HR) in man. However, it has not been reported whether injection or administration of phenylethylamine will cause an increase in plasma catecholamine concentrations.

Recent research has focused on the link between metabolic dysfunction in HFD-

induced obesity and GAPDH (Zheng et al., 2021). The previous study (Zheng et al., 2021) has shown that WEC and PHA were beneficial to NAFLD by decreasing the generation of methylglyoxal via increasing the expression of GAPDH. Bereta et al. (1995) reported that NO leads to increase in GAPDH mRNA levels making possible increased synthesis of this protein in murine microvascular endothelial cells (MME). Additionally, Stork et al. (2022) found that calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2) phosphorylation of GAPDH functions as an activation mark that bolsters GAPDH activity and subsequently promotes glucose catabolism despite the different opinions existing. Thus, in this model, it is proposed that the possible increases in plasma norepinephrine (NE) following treatment with phenylethylamine activate the α -adrenergic receptor on the surfaces of liver cells, activates a heterotrimeric G protein, whose membrane-anchored α subunit in complex with GTP diffuses laterally along the plasma membrane to activate the membrane-bound enzyme phospholipase C (PLC). Activated PLC catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) at its glycerophospho bond, yielding inositol-1,4,5-trisphosphate (IP₃) and 1,2-diacylglycerol (DAG). IP₃ induces an increase in cytosolic Ca²⁺ by opening IP₃-gated Ca²⁺ channels in the membrane of the endoplasmic reticulum. After Ca²⁺ binds to calmodulin, the Ca²⁺/calmodulin complex stimulates the activity of nitric oxide (NO) synthase, an enzyme that catalyzes formation of NO from O₂ and the amino acid arginine. In particular, the present author can infer that, with the activation of NO synthase, NO diffuses from the vascular endothelial cell into neighboring hepatocyte, where it triggers the transcription of GAPDH mRNA, which increases the synthesis of GAPDH, an enzyme of the glycolytic pathway. To my best knowledge, mechanism for upregulation of PC has not been linked to NE. The mechanism underlying for upregulation of PC by administration of PHA remains to be solved.

5. Conclusions

The present results, at least in the present model, demonstrated that WEC and PHA improved metabolic impairment in HFD-fed mice. The metabolomics analysis showed the contents of metabolic intermediates linked to glucose metabolism (glycolysis and gluconeogenesis), the TCA cycle, pyrimidine metabolism, arginine biosynthesis, and glutathione metabolism pathway were altered by WEC and PHA.

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Table 1. Identification of result of gel band with an observed difference in densitometry.

Band no. ^a	Sequences of matched peptide ^b	Protein identified	Uniprot ID	Fold change (log2FC)	
				HFD/HFD- WEC	HFD/HFD- PHA
Fr2A	GIVVGIK	Fructose- biphosphate aldolase B	Q91Y97	0.14	0.65
	VLAAVYK				
	LSFSYGR				
	ATQEAFMK				
	DSQGNLFR				
	ATQEAFMK				
	ELSEIAQR				
Fr2B	SMLR	Carbonic anhydrase 3	P16015	-1.23	-0.28
	GGPLSGPYR				
	YNTFGEALK				
	VVFDDTYDR				
	EPMTVSSDQMAK				
	GDNQSPIELHTK				
	GEFQILLDALDK				
Fr2C	NDYVK	Glutathione S- transferase P 1	P19157	0.44	1.12
	SLGLYGK				
	MLLADQGQSWK				
Fr2D	VIGVK	Aconitate hydratase	Q99KI0	1.69	1.37
	AIITK				
	DVILK				
	NINIVR				
	YDLLEK				
	VAGILTVK				
	DGYAQILR				
	EGWPLDIR				
	VDVSPTSQR				
	NTIVTSYNR				

Table 1. (continued)

Band no. ^a	Sequences of matched peptide ^b	Protein identified	Uniprot ID	Fold change (log2FC)	
				HFD/HFD- WEC	HFD/HFD- PHA
Fr2E	FMGK	Alpha-enolase	P17182	1.42	1.86
	GLFR				
	YDLDFK YNQILR				
	EAELELLK				
	TIAPALVSK				
	IEEELGSK				
	VNVVEQEK				
	SCNCLLLK				
	IGAEVYHNLK				
	GVSQAVEHINK				
	LMIEMDG TENK				
	LFVVK				
Fr9A	QDLLR VGDLSPK	SEC14-like protein 2	Q99J08	0.38	0.05
	QYYVR				
	SFDLQK				
	VLPALSPA				
	VGFGQCAGK				
Fr9B	FGPEPIISK	Fumarylacetoacetase	P35505	0.39	0.61
	AIDVEQGQTR				
	ASSIVVSGTPIR				
	ASLQNLLSASQAR				

Table 1. (continued)

Band no. ^a	Sequences of matched peptide ^b	Protein identified	Uniprot ID	Fold change (log2FC)	
				HFD/HFD-	HFD/HFD-
				WEC	PHA
Fr9C	LAEQYAK	Ornithine	P11725	0.71	0.39
	QSDLDTLAK	transcarbamylase			
	GEYLPLLQGK				
	GYEPDPNIVK				
	VLSSMTDAVLAR				
Fr13A	EYGVK	Carbamoyl-	Q8C196	1.95	2.18
	WDLDR	phosphate synthase			
	MSMANR				
	WFLYR				
	TSVNVVR				
	EIGFSDK				
	VAQAPWK				
	SFPFVSK				
	DILNMDK				
	LNEINEK				
	FLEEATR				
Fr17A	VVVS	Annexin A5	P48036	-0.63	0.22
	TLFGR				
	ADAEVLR				
	WGTDEEK				
	FITIFGTR				
	TPEELSAIK				
	VLTEIIASR				
	LYDAYELK				
	SEIDLFNIR				
	GTVTDFPGFDGR				

Table 1. (continued)

Band no. ^a	Sequences of matched peptide ^b	Protein identified	Uniprot ID	Fold change (log2FC)	
				HFD/HFD- WEC	HFD/HFD- PHA
Fr21A	VQQLVPK ALDFIASK	Alpha-actinin-4	P57780	0.69	0.96

^a Band numbers correspond to the gel in Figure 2.

^b Peptide sequences were confirmed by tandem MS spectra after database matching.

Table2. Immunoblotting analyses of protein levels in livers in HDF, HFD-WEC, and HFD-PHA.

Protein name	Uniprot ID	Fold change (log2FC)	
		HFD/HFD-WEC	HFD/HFD-PHA
Pyruvic carboxylase	E9QPD7	4.14	4.13
Glucokinase	P52792	0.47	0.58
Phosphofructokinase, liver type	P12382	0.0035	0.0061
Argininosuccinate synthase	P16460	1.09	1.25
Glutathione S-transferase A3	P30115	0.079	0.24
Pyruvate dehydrogenase E1 subunit alpha 1	P35486	0.11	0.062

Legends for Figures

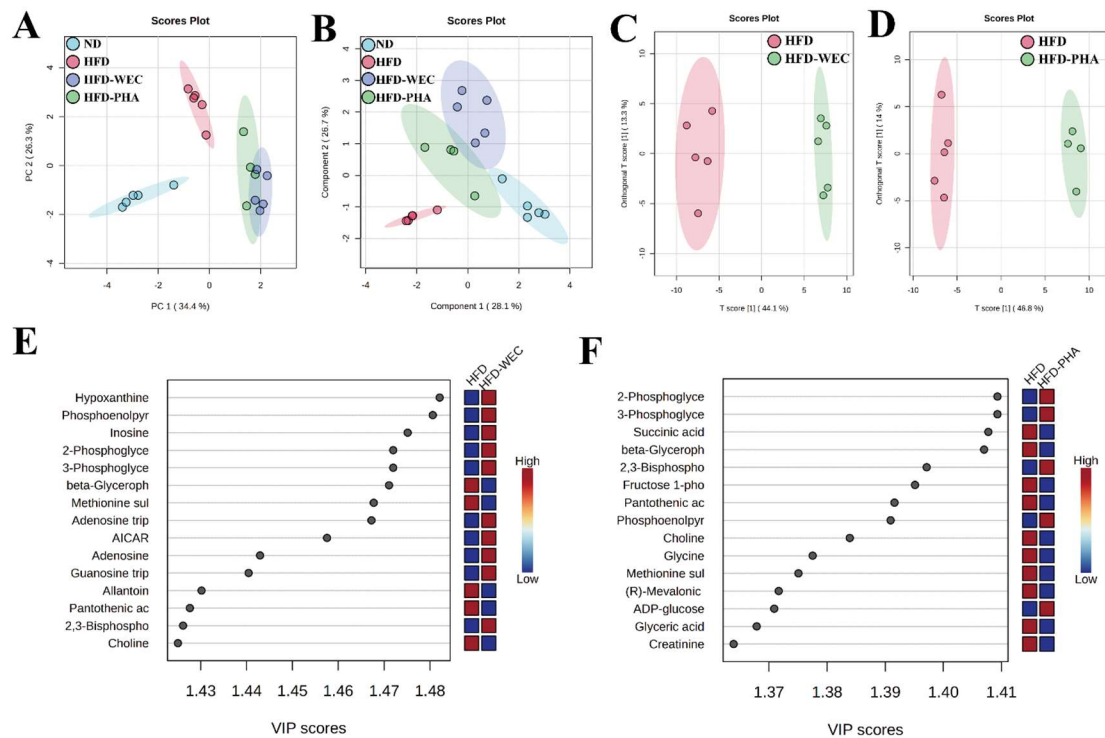
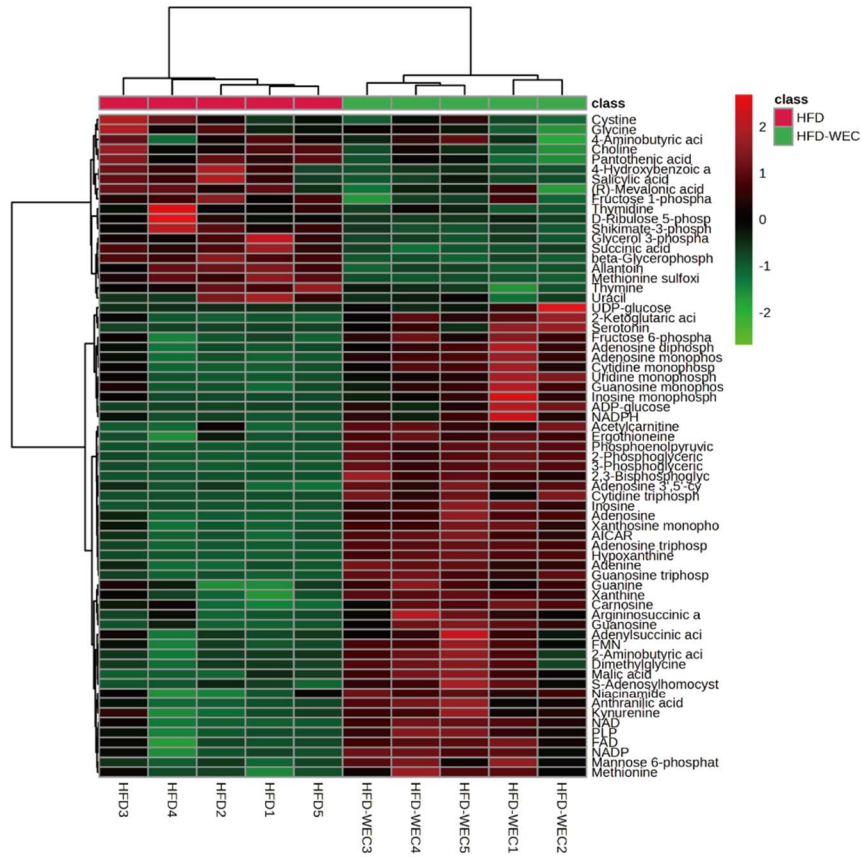


Figure 1. Analysis of metabolic profiles in the liver. (A) PCA score plot of the 135 metabolites among four groups; (B) PLS-DA score plot of the 135 metabolites among four groups; (C) OPLS-DA model between HFD and HFD-WEC groups; (D) OPLS-DA model between HFD and HFD-PHA groups; (E) Top 15 metabolites based on VIP scores from OPLS-DA model between HFD and HFD-WEC groups. The VIP score in x axis quantified the contribution. The color on the right represents the relative amount of each phenolic in each diet group; (F) Top 15 metabolites based on VIP scores from OPLS-DA model between HFD and HFD-PHA groups. The VIP score in x axis quantified the contribution. The color on the right represents the relative amount of each phenolic in each diet group.

A



B

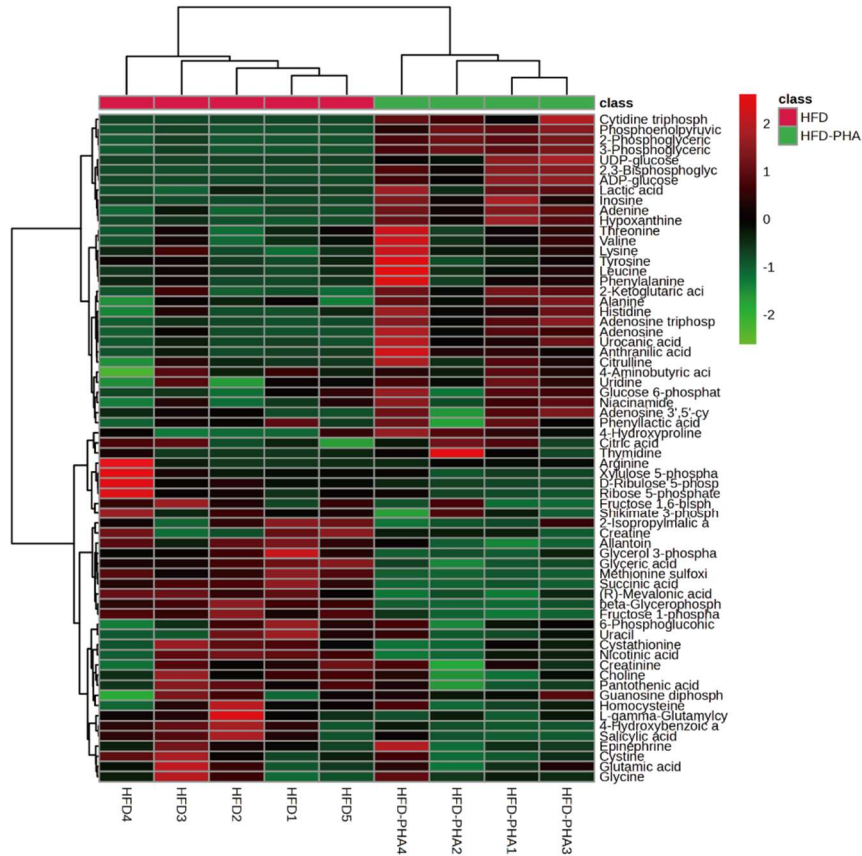


Figure 2. Heatmap visualization of significantly changed metabolites in HFD vs. HFD-WEC groups (A), and HFD vs. HFD-PHA groups (B). Each row represents a metabolite, and each column represents a sample. The red color indicates up-regulation and green represents down-regulation.

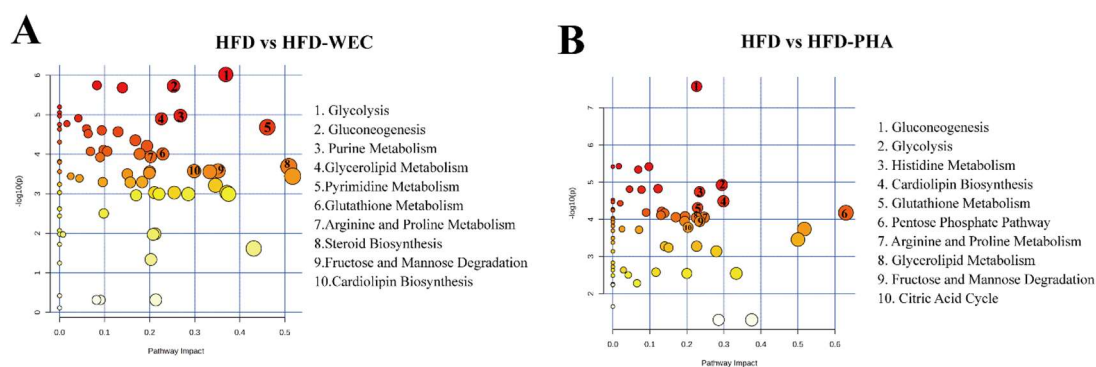


Figure 3. Pathway analysis of differentially expressed metabolites using MetaboAnalyst in HFD vs HFD-WEC groups (A) and HFD vs HFD-PHA groups (B). The pathway impact values (x-axis) represent the influencing factor of topological analysis, and the $-\log_{10}(p)$ (y-axis) represents the p value of the pathway enrichment analysis. Size of the nodes shows the influence factor of topological analysis, while color of the nodes indicates p value of the enrichment analysis.

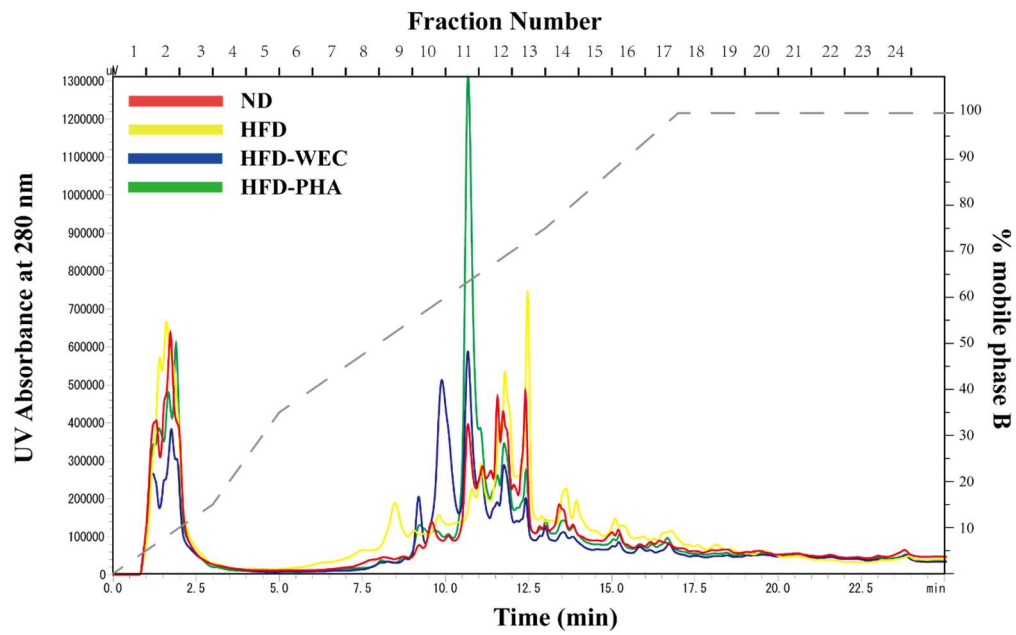


Figure 4. Overlap of four chromatograms for pooled liver tissue lysate from groups ND, HFD, HFD-WEC, and HFD-PHA. The protein was separated and eluted with a linear gradient on the Type-Q anion-exchange column. Absorbance at 280 nm was monitored and 24 fractions were collected from each chromatographic run.

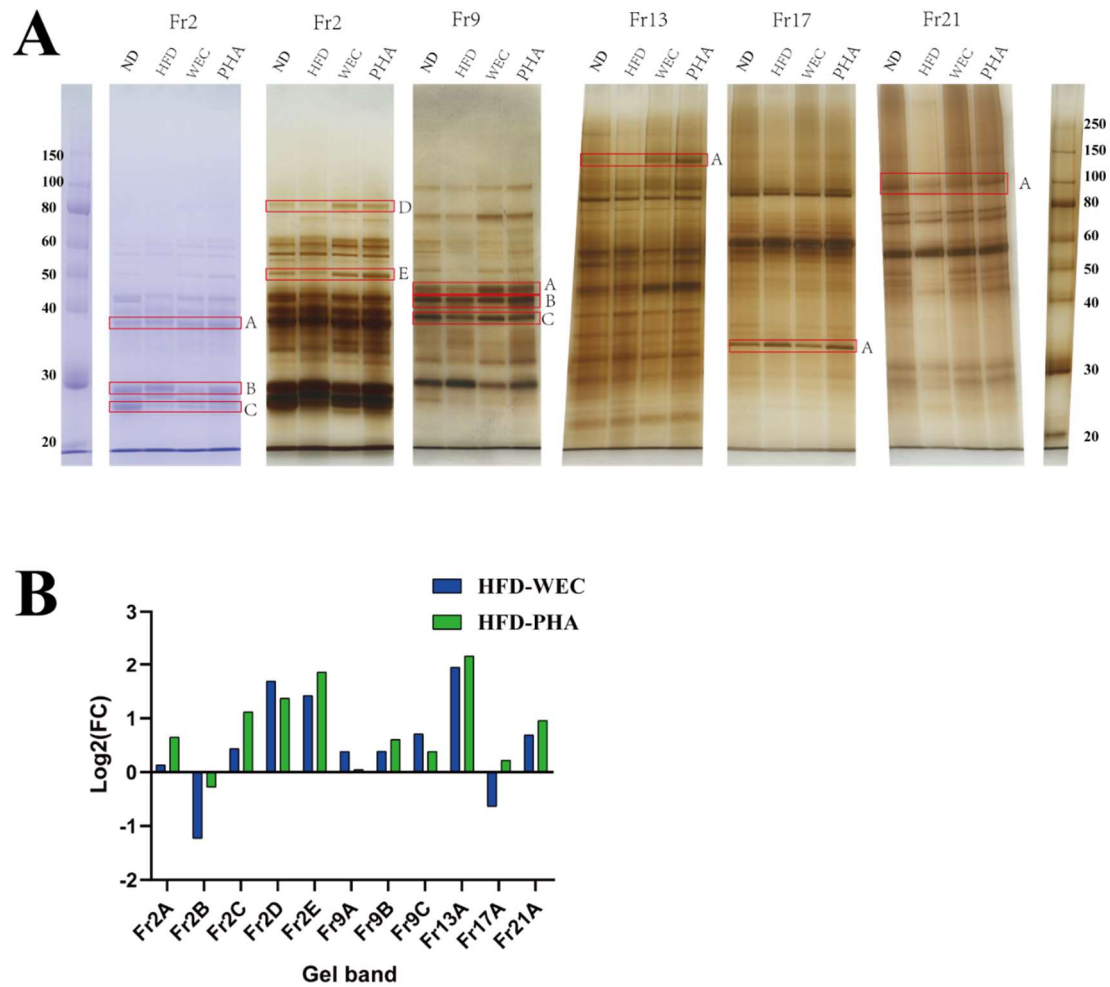


Figure 5. Comparison of mouse liver proteomes fractions via 1D SDS-PAGE separation. (A) Bands of interest, that are excised and extracted proteins digested with trypsin and subsequently analyzed by LCI-ESI-MS/MS, are boxed and labeled with letters for correlation with Table 1. (B) Differential gel band analysis. Using ImageJ densitometry software, differential gel band intensities compared to the HFD group were quantified to derive the relative log2FC increase and decrease. Quantitative densitometric analysis was performed on the selected bands based on their relative intensities.

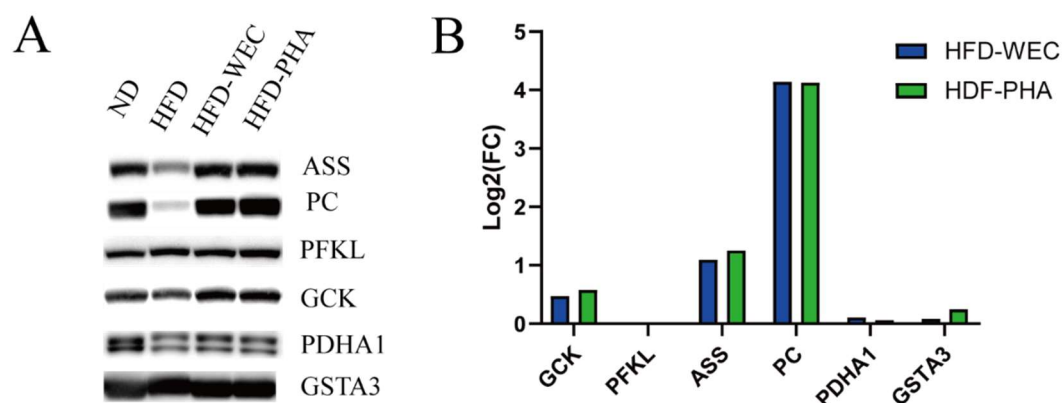


Figure 6. Comparison of mouse liver proteomes via immunoblotting.

(A) Representative immunoblotting analysis of GCK, PFKL, PDHA1, PC, ASS, and GSTA3. Abbreviations are: ASS: argininosuccinate synthase; PC: pyruvate carboxylase; PFKL: phosphofructokinase-1; GCK: glucokinase; PDHA1: pyruvate dehydrogenase; GSTA3: glutathione S-transferase A3.

(B) Differential protein levels analysis. Using ImageJ densitometry software, differential protein intensities compared to the HFD group were quantified to derive the relative log2FC increase and decrease.

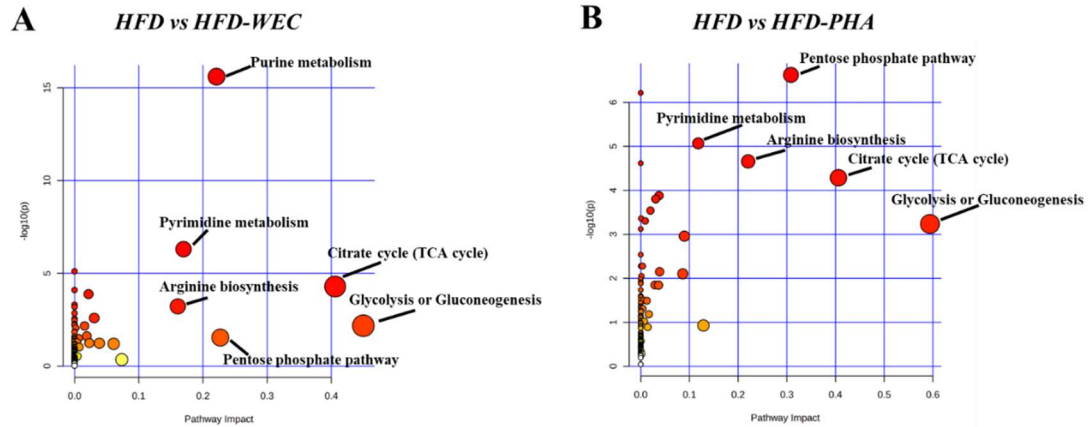


Figure 7. Combined multi-omics analysis using MetaboAnalyst. (A) Pathway analysis of differentially expressed proteins and metabolites between HFD and HFD-WEC groups; (B) Pathway analysis of differentially expressed proteins and metabolites between HFD and HFD-PHA. The pathway impact values (x-axis) represent the influencing factor of topological analysis, and the $-\log_{10}(p)$ (y-axis) represents the p value of the pathway enrichment analysis. Size of the nodes shows the influence factor of topological analysis, while color of the nodes indicates p value of the enrichment analysis.

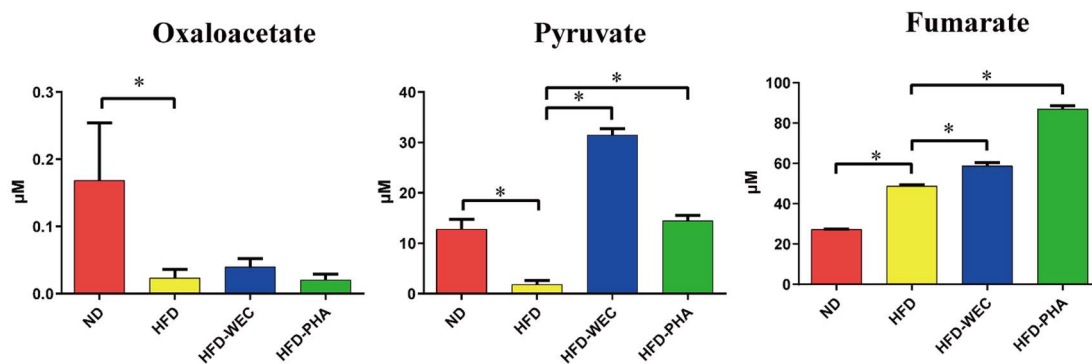


Figure 8. Concentrations of central carbon metabolism carboxylic acids in liver extract detected using 3-NPH derivatization method/reverse phase method. The data are displayed as mean $\pm$ SD, $n = 3$. * represents significant difference with $p < 0.05$ by Dunnett's test vs HFD.

Supporting information

Contents

Supplemental Table 1. The liver metabolome data reported in this study.

Supplemental Table 2. Significantly changed metabolites of HFD vs HFD-WEC.

Supplemental Table 3. Significantly changed metabolites of HFD vs HFD-PHA.

Supplemental Table 4. MetaboAnalyst Pathway analysis of HFD vs HFD-WEC.

Supplemental Table 5 MetaboAnalyst Pathway analysis of HFD vs HFD-PHA.

Supplemental Table 6. MetaboAnalyst result joint-pathway HFD vs HFD-WEC.

Supplemental Table 7. MetaboAnalyst result joint-pathway HFD vs HFD-PHA.

Supplemental Figure 1. Heatmap of the global 135 metabolites for all groups.

Supplemental Figure 2. The gels were visualized with CBB and silver stain.

Adenosine triphosphate	297575	317527	316456	385074	463308	127139	164541	290853	56434	151281	1393710	1242637	1327021	1367719	1483961	904754	468682	1145433	1125845
Adenylsuccinic acid	970466	1304631	533917	729594	2839298	797866	970920	1547535	351346	932783	1939422	1251447	1451056	1878213	3302835	963085	1037197	1837475	1269696
ADP-glucose	46946	57898	38569	29499	34146	NA	NA	NA	NA	NA	123245	90113	53443	17154	49372	22172	8152	22654	14678
AICAR	210249	253519	168387	118521	220913	62929	115660	184739	79956	118960	403455	360362	432734	474698	542274	219635	272025	335577	372295
Alanine	816835	838446	710814	910266	1027139	714208	698603	714680	652615	660133	857800	718551	720600	833427	713781	750866	706762	765700	754937
Allantoin	554862	637591	432624	453477	815295	1313577	1223175	777503	1195461	1049214	383430	459283	371596	516660	508111	417912	559522	541468	922273
Anthratic acid	270756	304547	269197	332247	212185	157195	118084	329790	82679	182393	358711	423255	610399	712222	814376	640578	583621	475178	1380165
Arginine	27746	28986	30440	24770	19876	12424	11773	18521	83809	14893	25518	26307	16940	30430	21248	22022	23857	22244	15844
Argininosuccinic acid	2175322	1867411	1534624	1451245	5186376	4025699	2881667	5029223	8552964	4666591	12135717	9694464	8726849	20949043	16253798	14061079	14203689	12139358	13751530
Asparagine	38568144	41128756	41581532	42261737	42699231	46381304	42452881	49721177	47993563	43903043	46594937	47913871	48105895	56301798	53548072	48356824	48883847	52883854	69893955
Aspartic acid	11936713	8874950	13294101	11279429	10768010	8357241	6174952	9671155	5503711	8663800	5327510	5184968	6647015	10577506	10015452	8045527	7010002	10928275	17534667
Asymmetric dimethylarginine	1.62E+08	1.7E+08	2.12E+08	2.2E+08	1.66E+08	99243828	1.2E+08	1.66E+08	1.09E+08	1.16E+08	98960081	88189197	115201123	202605852	201564428	160680368	123963581	167743479	278746195
Camitine	23977262	20582681	24667290	18565726	22106030	19911826	12406724	13423993	11946013	19376435	16191417	16956846	18476730	18419941	18421104	15415466	9912241	18235460	31318290
Camosine	71493521	68934231	72886383	68493358	64663696	22394628	27995572	47535661	56256248	27333579	80005597	71587324	50984556	75414751	71681834	56784731	41547678	49901770	101249932
Cholic acid	399664	385917	333921	572671	1145990	227501	212103	421266	107511	288659	190100	706939	404318	30604	96647	930477	956047	140286	419490
Choline	48258467	51174420	62119927	56181768	56900012	42349883	37423074	49489525	34494624	43116850	27404937	22165113	28633150	37288617	31453355	29330683	26710038	36801038	40806401
Citric acid	5059684	17883677	13182110	13455605	2424133	1687921	1495625	2246326	2168027	1117327	3077120	2899963	1580964	2855599	2053587	2205991	2351700	1542264	1731235
Citrulline	16007302	16509185	11895192	15799370	10807488	5176525	5599386	6707056	3641354	5057504	6509422	9173519	6107087	7209848	8981656	7403002	5268009	6485187	8954789
Creatine	60427537	34318547	70195969	62370175	73215537	84731409	61042483	56471190	86163553	89595789	91212221	81043833	61858114	44350519	56373489	67279360	67567959	59155271	67581769
Creatinine	14658802	12809171	21138292	15564533	11941583	8723155	8390761	9206854	7303442	9452969	10359676	9364855	6358445	7659790	8815584	8660130	6718585	7925607	9109432
Cystathionine	30761738	30131416	20152324	22591006	28020525	26430006	27237055	29772125	20047231	22899015	30428572	25237600	30439320	28368053	35069423	23808683	19195514	22138035	18738317
Cysteine	158876	234906	180607	310096	214672	22928	44380	71958	27826	22949	30340	29743	31933	53813	83562	26267	29056	57521	86780
Cystine	46671666	53202915	44840279	72356326	52101175	9376459	14071536	24130163	19336507	11610365	8333239	6048246	6721458	11726831	15354102	8280390	6988213	10831934	17602003
Cytidine	24474780	33486017	44190648	21628174	18904670	15198584	13675153	15090974	80183089	29931125	20594472	10145519	11430586	33703730	28954980	25803460	19574215	15514854	49102984
Cytidine monophosphate	864490	105180	79838	54867	109622	30855	32788	92209	26540	40993	186177	106746	88555	137636	130556	105097	69846	126671	108260

Cytidine triphosphate	NA	NA	NA	6330	NA	NA	NA	NA	NA	6483	16412	16113	10255	15357	2756	5281	9203	6180	
Cytosine	2128725	2909827	3684669	1955115	1524262	1066807	1171294	1355587	6618795	2305171	1846547	900866	1114218	2950348	2678230	2606217	2023158	1660366	4223236
Dihydroxyacetone phosphate	109876	95486	101151	82534	355550	72226	115613	335741	83824	101272	456928	321626	305044	213046	152411	124709	150516	210865	149904
Dimethylglycine	6077695	64476328	61745258	47625644	56916263	36305766	36348429	36093128	30707905	35145112	51071949	34947394	50970104	54173198	56740749	49326852	51660678	36118683	64384687
DOXP	31862	34155	35599	33620	125151	23270	82028	365883	115768	61138	252477	189882	218816	229807	96950	129007	263387	200684	110589
Epinephrine	5121886	7042734	7741662	7627926	6948540	3569646	4053320	5354107	3318015	2793673	2312546	1321046	1806273	4073035	5013046	3082044	2155923	2906859	6314356
Ergothioneine	80748395	98146002	1.11E+08	83203534	1.79E+08	7045564	10899523	7320538	3085053	7802460	19714007	16899328	17559186	19481523	16092477	12088908	14048423	15442351	14089002
FAD	5618840	6569343	5086990	5995345	6245777	3521452	3707728	4956772	1889080	3658727	7570593	5571004	6404104	6941160	6931031	5999112	4608232	6767739	6137919
FMN	304447	323785	341948	335047	253957	175594	200950	241753	134139	189055	327905	245160	324133	326827	403598	231103	191375	339329	295586
Folic acid	787888	1066576	1411482	985751	998263	586899	231680	269886	237542	462688	748578	257913	376178	809810	782096	420357	294120	398302	883808
Fructose 1,6-bisphosphate	25912	35299	33671	41179	126110	30401	67064	114886	75022	74986	112282	26091	24612	28617	37695	15259	81408	16847	25903
Fructose 1-phosphate	21867	22848	24175	37390	84129	34981	48825	38230	41449	41687	41417	24864	19972	28516	28652	17432	19972	19904	25924
Fructose 6-phosphate	49273	41375	47362	62425	134051	64667	57297	97008	35666	50634	155878	119580	112986	143015	105411	84156	49259	108947	108841
Glucose 6-phosphate	1379825	1586052	1790222	1780419	2401094	1534604	1186220	1380837	1325920	1686535	1928535	1406066	1375308	2055894	2220193	1796554	1168704	1746800	2000997
Glutamic acid	15488146	17934709	15758163	19178875	14869054	5246976	8047354	10926347	6603498	5789080	6009578	3883329	5136577	6919560	7724990	5921954	4531757	7486312	7818866
Glutamine	112285	102242	82171	146215	255646	269455	299176	241488	240486	248880	529386	464421	440671	373896	351481	387921	398912	491746	242290
Glutathione	2273548	2446892	1594068	1258850	3384548	3906733	11470201	3166123	3881522	2355944	7348667	2134190	9337310	7016719	17214462	4248759	12693465	11244034	4697566
Glyceric acid	92498	74022	127902	159045	120507	116614	105170	93901	94186	123269	116726	94671	69718	71783	57437	65626	51982	68006	70919
beta-Glycerophosphoric acid	74151	65551	81228	134221	142196	174512	224585	178300	163764	171077	68952	48612	55105	62019	45087	69777	72630	85447	76612
Glycerol 3-phosphate	1104152	1170984	1738368	1460520	2766356	2888117	1769624	1311951	1234073	1557716	645254	390338	535242	573312	408686	560888	650081	1014281	548327
Glycine	4168018	43273195	39377030	40560091	39665246	27559163	31564374	34849587	29466922	28186887	25584954	23671208	28782938	29395668	27992583	29516252	28782107	29166496	32471548
Guanine	7873	4642	7528	8319	4043	383	248	4925	3386	2550	4796	5766	5562	7889	6202	3244	1165	6278	5361
Guanosine	48144296	99247105	55639111	59759491	28191453	2822248	3245853	7187307	20048118	6340776	52095708	39385602	28948550	56185809	61640112	40110636	28078597	23741272	54345560
Guanosine diphosphate	257423	235144	227948	334423	306283	215372	352260	394685	155396	306807	446255	252311	416007	396614	437522	287876	279819	365540	319760

Guanosine monophosphate	932649	1185073	771432	1018433	339025	37361	67742	184895	60639	74884	364460	230183	122481	206092	216702	175793	98017	183955	182517
Guanosine triphosphate	38435	66671	49200	46667	64440	30991	46122	64123	27840	39904	167674	218222	221771	235480	199751	138845	56229	170123	157350
Histamine	3944763	1710191	1588167	1713812	1465454	564119	150196	587065	603076	448756	689585	333991	679861	1475647	1833470	1016539	639557	98096	3746559
Histidine	125182	129020	104035	121549	159255	101103	102456	129865	88099	112150	150842	93714	120779	173878	149467	128008	119292	147563	160783
Homocysteine	213972	227337	182190	167747	250789	165118	230956	180343	135427	170901	150375	139782	174493	144224	174504	146009	134138	159138	192355
Hypoxanthine	3455677	3270295	4883196	3598988	3004404	88457	393271	1653194	658754	555030	13392321	13370272	12502540	14680683	15895648	9782083	3650013	6761658	7486749
Inosine	32675198	47559890	41427592	30372199	11577907	58466	120741	172784	1431119	608255	35074078	23906689	23719240	26286049	41084383	22179344	8127775	9329631	18255672
Inosine monophosphate	120417	172097	77094	124385	34675	NA	NA	20747	NA	NA	78221	34027	12681	22957	33756	11901	5830	26858	12637
Iscitric acid	4449866	15769434	11197562	11893527	2154476	1492971	1269074	2012415	1976821	1043729	2713640	2623879	1426539	2329065	1714942	2037465	2101839	1406362	1608858
Isoleucine	81867947	72341668	89166933	82598631	81167704	70407673	52651702	91164784	62159014	78624037	62514563	60609725	73046552	111717068	104793167	81412013	64019451	99605949	137965311
Kynurenine	3177568	4085628	1853666	2409975	4204780	1769513	1276888	3320373	900245	1934991	2950265	3186929	3463590	3560036	4770487	3702139	2630614	2090773	10213422
Lactic acid	12199988	11832735	10839500	11838459	11069293	8872920	9302237	8149069	8473091	8764414	12193388	9525219	9924792	11227904	12663056	11650925	9094217	11432629	12641998
Leucine	6121360	5880199	6120454	7222153	8418092	5381342	5645954	6714994	5683121	5858478	6772801	4776711	5255866	8380212	6704259	6283194	5795762	7038192	10100184
Lysine	465186	466359	441095	557814	590192	303724	336740	465615	367767	361274	380935	233342	301789	524663	429709	380660	382208	435800	592461
Malic acid	32215690	30159525	18502344	25028425	46200660	50287534	42389933	43407150	43521062	47659428	63885595	56161036	63351991	70651876	74086187	66298401	70354246	64329681	71851351
Mannose 6-phosphate	6694	6524	9253	6912	16832	5497	9095	9459	6413	8138	24724	12500	20036	23169	14439	17063	13302	18206	24755
MEP	17952	7592	17893	14359	24741	15388	12863	26023	8338	8517	18187	22349	27215	15344	13052	15688	14139	12006	17931
Methionine	203961	201827	233810	269739	520578	115546	215660	331722	204463	198539	445963	305058	338550	547561	429981	413473	391755	461002	618125
Methionine sulfoxide	56498556	63689239	56295922	47312905	14681204	93513865	63842532	53763301	76364166	74966524	15202060	12560414	17973158	14715201	17805774	22708529	18624671	20450662	19739518
(R)-Mevalonic acid 5-phosphate	37262	46934	45883	46186	60599	65931	60427	66716	66367	54728	62665	45626	48744	55774	56420	41153	46445	50518	41676
NAD	2076911	2323038	2240136	2134094	2557904	956181	998021	2865355	445036	1083263	4165804	3261176	3777285	4780021	4575588	3586542	2563578	3838861	4122581
NADH	1069212	1121874	952148	898392	552274	135614	233788	294599	91550	148930	321967	276191	237800	211076	337774	250469	132186	241872	257219
NADP	1060833	1072034	861238	1220501	1113499	574387	501164	847442	204037	518592	1373722	850252	1423424	1443857	1275972	1049274	718597	1288224	917441
NADPH	221966	199840	122940	157808	10694	3015	6181	12668	3621	4528	46652	20498	22164	10907	24884	20525	14333	23193	12379
Niacinamide	63050794	65877145	71589601	69393544	64175247	57304087	48556426	71626845	46390733	71046589	79429357	83483123	91345597	84249529	89429724	75481039	54278970	79775919	87434138

Nicotinic acid	4733233	3739493	3276756	3743252	3418562	2098675	2184061	2086576	1191173	1952324	1844028	1800684	1807727	1187356	1287778	1523032	1138800	1491493	1059104
Ophthalmic acid	48010	41607	60876	36909	40392	97021	117668	100379	151645	127900	183470	169190	187307	58525	78253	220157	171898	77079	169497
Ornithine	48570468	38008399	46015996	41033551	44581190	36852146	27053048	42991516	38068732	40110218	33949373	34049546	39168612	56076915	53037288	43419235	34162478	50392905	74653574
Orotic acid	248443	172604	311027	468130	519113	176127	167123	257174	223932	213477	242324	226105	191259	361100	321206	250341	501871	214933	236859
Oxidized glutathione	3235387	2243236	1569959	913430	2671639	16668335	14598564	12950119	9367021	12715327	17720812	16993818	22425934	16207297	16472581	22696234	18124844	16422843	13167612
Pantothenic acid	1.47E+08	1.63E+08	1.78E+08	1.77E+08	1.4E+08	1.91E+08	2.22E+08	2.39E+08	1.65E+08	2.15E+08	113466827	93809076	128218857	165736530	160666677	152105752	125314553	162401065	196555865
Phenylalanine	173357	179620	169344	199563	317960	182838	182945	229472	202330	194784	230730	143196	165649	289691	228183	231887	185469	238783	352072
Phenyllactic acid	39938	41873	28764	34255	19481	27546	21412	23048	15699	18218	43650	42034	23340	23144	27709	28039	11333	21812	28571
Phosphoenolpyruvic acid	2292	1464	2238	1879	19809	820	NA	4521	NA	1354	84211	74949	76578	56875	77365	46287	50417	57535	31438
PLP	524382	696583	560765	481149	989346	700633	643768	928587	475131	694718	1173439	1056644	1178488	1509927	1468678	1386077	1051697	1250546	1577001
Proline	50253892	47213783	62501763	39650999	59767166	47871318	33821017	59526403	38459793	56715968	42616517	41608196	49809208	75917475	73844300	57477542	43265624	68135789	94542272
Ribose 1-phosphate	556871	769749	547000	665831	259427	53399	69520	100867	154101	65133	178966	71591	101594	104239	141740	90711	75522	80718	170610
Ribose 5-phosphate	477646	905489	1041359	793397	779445	215012	347369	318089	731980	309802	253693	166366	197601	208170	261717	167332	181504	148951	341980
D-Ribulose 5-phosphate	132640	210721	261305	239583	207844	82742	133742	100089	320591	87614	72576	37866	49111	43144	47049	34194	37941	29511	65185
S-Adenosylhomocysteine	21945223	24272895	21259385	28159577	11729411	7029061	7941820	6280852	6271541	5616545	12884186	9040046	10970823	11723629	15494102	10256735	5035693	7818012	11265734
S-Adenosylmethionine	4754623	5691182	5248928	11347412	3446521	3117809	6827039	5724470	3509629	4271256	7113216	17138728	5778614	4275272	4224752	4626719	2616950	8502578	2496083
Salicylic acid	87331	129895	62830	37958	72579	46893	85438	61158	50091	15054	10373	10324	6532	12714	25687	12302	14379	12502	39023
Sedheptulose 7-phosphate	155801	185161	299008	201871	297630	158406	94745	98116	135779	160434	129047	94039	94591	150723	181437	180893	159353	136220	244997
Serine	53129655	47208653	65268574	54417678	48089996	42285961	29371289	48310500	33874421	47639270	31934684	35480769	42262674	60320364	55388847	43831822	35231030	54082399	79120152
Serotonin	15959	33981	57918	53929	90511	NA	NA	NA	NA	NA	1024843	1052338	333913	583782	121491	26649	124182	224679	NA
Shikimic acid	692304	707893	562019	806640	473999	82587	68947	31851	75793	58818	804024	52404	135605	35259	350965	350777	96601	312488	217863
Shikimate 3-phosphate	197142	174596	141554	208835	113414	112238	134995	101278	172151	123896	83294	72011	84135	74995	74060	105013	143804	81638	56383
Succinic acid	4063476	4364797	5033884	5040491	3358066	4369025	3186327	3165244	2560760	2715390	812751	1116624	1024700	319401	774895	507596	282092	371445	236397
Symmetric dimethylarginine	56414573	47027107	69525742	70929622	41266013	9028269	14017248	27177046	12731691	12412982	8097500	8126037	9447224	29710719	26374188	21571256	16208852	20216390	48909530

Taucohic acid	20901225	19813184	17959657	20164400	43078335	16027011	13626512	15293877	12712419	10415486	7993673	36073219	15573780	2930209	9795181	51597854	61899279	9099631	35930769
Threonine	25222692	21705579	29129168	25085383	23056811	20039162	15422947	23810531	16851725	23079033	17762201	17990259	21434099	29092087	27688990	23126551	18366065	25226053	36047200
Thymidine	107973	100374	131396	126189	110959	115604	113978	109261	340078	169076	37303	40876	64067	134290	88812	276560	933843	69147	297951
Thymidine monophosphate	NA	NA	NA	NA	NA	NA	11737	11597	60040	10702	6099	NA	NA	13072	NA	39160	129339	NA	26661
Thymine	NA	17017	NA	33119	50229	87658	99006	61268	69413	117832	NA	31125	52960	45385	39403	50136	81110	76991	56240
Tryptophan	10024401	8333371	11770814	8932087	12252738	7802493	6626472	12894691	8798985	10220172	8762429	8487861	9793222	15758135	13903690	11515356	9556178	14432083	21958094
Tyramine	40507	56693	63585	56964	50995	NA	NA	NA	NA	NA	NA	25670	NA	NA	NA	NA	NA	NA	NA
Tyrosine	2824765	3056620	29152272	3461812	3947635	2641245	2784512	3422717	3388805	2993286	3049753	2102009	2395105	3994372	3281999	2987975	2620632	3419742	5283298
UDP-glucose	47397	213013	180022	143466	135318	NA	NA	NA	NA	NA	269822	834243	121602	17385	71123	23120	6047	26608	8680
Uracil	1072719	1056312	1376946	1091553	2242216	3479455	3303275	2155555	2127867	2785891	1785797	2115994	2189672	2299566	2434465	2210159	2134438	2515269	2887543
Uric acid	6952120	6095325	7498421	8615189	7556573	477188	3772568	10019936	1929284	4168617	5800755	5088998	6373000	2092270	1871383	1878232	5629045	6341095	NA
Uridine	278623	382698	352595	489484	379646	327424	289414	344886	292588	333258	485086	261896	295251	396161	324310	349785	322227	336066	342138
Uridine diphosphate	NA	NA	NA	NA	9289	9451	10144	10618	33051	18941	17479	14523	7339	12631	14240	21837	17743	8715	15023
Uridine monophosphate	126500	150656	92732	139971	71976	18202	19133	50131	20991	21987	114248	100320	46998	59416	64885	59846	39263	69101	53992
Urocanic acid	2436377	1462367	1222290	957864	3870764	1721680	1448439	3134281	891452	1145010	1986972	1372164	2648215	7541333	6149721	5333730	4236637	8320216	11354237
Valine	40493822	33131135	42913162	40816031	43771606	43880342	35764924	52922530	39017965	47783944	38594192	37227725	45341473	60970636	56251247	51556932	43413875	57626404	79417584
Xanthine	32879328	32814852	36457206	35697976	31296179	2156536	12243484	29494053	19217634	16117797	44316950	40431860	47816682	47827676	49640169	49506832	41572782	47031938	44739783
Xanthosine monophosphate	30918	34467	19779	36688	19177	1461	2556	7344	NA	2583	17728	12408	14191	13591	18432	9544	4522	11585	8433
Xylulose 5-phosphate	42228	80039	97448	76127	82286	36230	33489	46286	100558	37205	46464	30893	31038	39335	34512	23712	17411	21614	37622

Supplemental Table 2. Significantly changed metabolites of HFD vs HFD-WEC.

Metabolites	VIP	<i>p</i> .value
(R)-Mevalonic acid 5-phosphate	1.193892	0.009515
2,3-Bisphosphoglyceric acid	1.426167	7.16E-06
2-Aminobutyric acid	1.142017	0.006786
2-Ketoglutaric acid	1.177428	0.007129
2-Phosphoglyceric acid	1.471979	1.82E-06
3-Phosphoglyceric acid	1.471979	1.82E-06
4-Aminobutyric acid	1.034498	0.029391
4-Hydroxybenzoic acid	1.235246	0.006407
Acetylcarnitine	1.129154	0.005103
Adenine	1.415488	0.000107
Adenosine	1.442951	2.87E-05
Adenosine 3',5'-cyclic monophosphate	1.062776	0.012675
Adenosine diphosphate	1.24922	0.004015
Adenosine monophosphate	1.350412	0.000708
Adenosine triphosphate	1.467242	1.64E-06
Adenylsuccinic acid	1.122711	0.018786
ADP-glucose	1.297995	0.001072
AICAR	1.457545	8.25E-06
Allantoin	1.430197	0.000157
Anthranilic acid	1.369105	0.000535
Argininosuccinic acid	1.278298	0.00244
beta-Glycerophosphoric acid	1.471076	9.26E-06
Carnosine	1.121565	0.01511
Choline	1.42505	1.75E-05
Cystine	1.078147	0.013216
Cytidine monophosphate	1.31994	0.002011
Cytidine triphosphate	1.389959	4.94E-05
Dimethylglycine	1.059932	0.013557
D-Ribulose 5-phosphate	1.126602	0.016625
Ergothioneine	1.20694	0.002866
FAD	1.229063	0.00405
FMN	1.299566	0.001138
Fructose 1-phosphate	1.165196	0.011281
Fructose 6-phosphate	1.216617	0.004823
Glycerol 3-phosphate	1.37853	0.000492
Glycine	1.253799	0.005786
Guanine	1.126589	0.025589
Guanosine	1.376813	0.00031
Guanosine monophosphate	1.164275	0.014419
Guanosine triphosphate	1.440458	5.74E-06
Hypoxanthine	1.482115	1.02E-06

Inosine	1.475127	7.27E-07
Inosine monophosphate	1.215133	0.008385
Kynurenine	1.219025	0.008526
Malic acid	1.202528	0.002381
Mannose 6-phosphate	1.246971	0.002359
Methionine	1.234745	0.005144
Methionine sulfoxide	1.467729	6.71E-06
NAD	1.360878	0.000708
NADP	1.252543	0.003159
NADPH	1.201398	0.006998
Niacinamide	1.098663	0.019009
Pantothenic acid	1.427647	3.68E-05
Phosphoenolpyruvic acid	1.480612	1.46E-06
PLP	1.399876	0.00012
S-Adenosylhomocysteine	1.213119	0.002001
Salicylic acid	1.260362	0.004267
Serotonin	1.265066	0.001724
Shikimate-3-phosphate	1.284257	0.003738
Succinic acid	1.397147	0.000218
Thymidine	1.101981	0.017052
Thymine	1.236495	0.005897
UDP-glucose	1.097143	0.01407
Uracil	1.086139	0.037472
Uridine monophosphate	1.204243	0.007736
Xanthine	1.230231	0.005941
Xanthosine monophosphate	1.358111	0.000649

Supplemental Table 3. Significantly changed metabolites of HFD vs HFD-PHA.

Metabolites	VIP	<i>p</i> .value
(R)-Mevalonic acid 5-phosphate	1.371682912	0.000341
2,3-Bisphosphoglyceric acid	1.397133854	7.85E-05
2-Isopropylmalic acid	1.224413697	0.004915
2-Ketoglutaric acid	1.054331302	0.031558
2-Phosphoglyceric acid	1.409311816	2.25E-05
3-Phosphoglyceric acid	1.409311816	2.25E-05
4-Aminobutyric acid	1.257896116	0.002266
4-Hydroxybenzoic acid	1.182500469	0.009885
4-Hydroxyproline	1.124369041	0.017987
6-Phosphogluconic acid	1.181334715	0.006626
Adenine	1.211114551	0.00792
Adenosine	1.006817981	0.046387
Adenosine 3',5'-cyclic monophosphate	1.151967827	0.010973
Adenosine triphosphate	1.287363841	0.002649
ADP-glucose	1.370905439	0.000258
AICAR	0.985187995	0.041298
Alanine	1.295966235	0.001735
Allantoin	1.277461501	0.00232
Anthranilic acid	1.121122623	0.018512
Asparagine	1.193731081	0.009119
beta-Glycerophosphoric acid	1.407010544	3.38E-05
Choline	1.383904804	9.00E-05
Citric acid	1.029114794	0.047788
Citrulline	1.000922117	0.032251
Creatine	1.207803611	0.006728
Creatinine	1.36392821	0.000144
Cystathionine	1.356089482	0.000259
Cystine	0.56832089	0.004919
Cytidine triphosphate	1.295324917	0.000836
D-Ribulose 5-phosphate	1.21418135	0.008373
Epinephrine	1.131442597	0.013701
Fructose 1,6-bisphosphate	1.238972037	0.006327
Fructose 1-phosphate	1.395126355	7.96E-05
Glucose 6-phosphate	1.135682907	0.011242
Glutamic acid	1.294732525	0.00173
Glyceric acid	1.367893818	0.000141
Glycerol 3-phosphate	1.259142242	0.002632
Glycine	1.377520898	0.000234
Guanosine	0.973267163	0.040351
Guanosine diphosphate	1.087214601	0.017329
Histidine	1.276486983	0.002004

Homocysteine	1.264864701	0.002123
Hypoxanthine	1.312104495	0.00106
Inosine	1.342650564	0.000444
Lactic acid	1.090416914	0.020516
Leucine	1.169533967	0.010432
L-gamma-Glutamylcysteine	1.182246915	0.010192
Lysine	1.248556533	0.004614
Methionine sulfoxide	1.375073846	0.000151
Niacinamide	1.20219753	0.004362
Nicotinic acid	1.312747914	0.000614
Pantothenic acid	1.391596667	4.23E-05
Phenylalanine	1.125093298	0.016073
Phenyllactic acid	1.009236381	0.030507
Phosphoenolpyruvic acid	1.390933854	6.15E-05
Ribose 5-phosphate	1.158998395	0.015117
S-Adenosylmethionine	0.998636381	0.040773
Salicylic acid	1.169869473	0.01113
Shikimate 3-phosphate	1.205906851	0.008956
Succinic acid	1.40773735	1.92E-05
Threonine	1.048186041	0.025916
Thymine	1.215081591	0.005476
Tyrosine	1.136253199	0.015764
UDP-glucose	1.275410005	0.00256
Uracil	1.150579313	0.010381
Uridine	1.343851597	0.000527
Urocanic acid	1.129454384	0.015397
Valine	1.076061505	0.02244
Xylulose 5-phosphate	1.189113727	0.010889

Supplemental Table 4. MetaboAnalyst Pathway analysis of HFD vs HFD-WEC.

		Total Cmpd	Hits	Raw p	#NAME?	Holm adjust	FDR	Impact
1	Glycolysis	20	6	9.55E-07	6.0199	8.41E-05	4.54E-05	0.36881
2	Gluconeogenesis	30	8	1.87E-06	5.7285	0.00016069	4.54E-05	0.25292
3	Purine Metabolism	63	17	1.06E-05	4.9765	0.000855	0.000101	0.2683
4	Glycerolipid Metabolism	23	6	1.26E-05	4.8996	0.00098282	0.000101	0.22587
5	Pyrimidine Metabolism	54	10	2.06E-05	4.6859	0.0015251	0.00012	0.461
6	Glutathione Metabolism	19	5	9.82E-05	4.0081	0.0060857	0.000308	0.22903
7	Arginine and Proline Metabolism	48	9	0.000116	3.937	0.006937	0.000347	0.2023
8	Steroid Biosynthesis	43	5	0.000204	3.6906	0.011418	0.000544	0.50877
9	Fructose and Mannose Degradation	28	8	0.000263	3.5798	0.014474	0.000641	0.35196
10	Cardiolipin Biosynthesis	11	3	0.000266	3.5751	0.014474	0.000641	0.2987
11	Glycerol Phosphate Shuttle	8	1	0.000277	3.5581	0.01456	0.000641	0.33333
12	De Novo Triacylglycerol Biosynthesis	9	1	0.000277	3.5581	0.01456	0.000641	0.2
13	Nucleotide Sugars Metabolism	16	3	0.000359	3.4454	0.017214	0.000751	0.51724
14	Pentose Phosphate Pathway	27	6	0.000609	3.2151	0.024262	0.000993	0.34604
15	Inositol Phosphate Metabolism	22	2	0.000932	3.0306	0.031675	0.001323	0.37157
16	Inositol Metabolism	28	2	0.000932	3.0306	0.031675	0.001323	0.25459
17	Trehalose Degradation	11	2	0.000932	3.0306	0.031675	0.001323	0.21053
18	Lactose Synthesis	14	4	0.001009	2.9961	0.031675	0.001376	0.22059
19	Glucose-Alanine Cycle	9	1	0.001016	2.9931	0.031675	0.001376	0.375
20	Malate-Aspartate Shuttle	7	1	0.001016	2.9931	0.031675	0.001376	0.28571
21	Vitamin B6 Metabolism	15	1	0.01031	1.9868	0.17526	0.01234	0.21199
22	Folate Metabolism	24	4	0.010657	1.9724	0.17526	0.01234	0.20762

23	Phosphatidylinositol Phosphate Metabolism	14	3	0.024144	1.6172	0.26558	0.027239	0.43089
24	Pterine Biosynthesis	18	3	0.045977	1.3375	0.45977	0.051215	0.20279

Supplemental Table 5. MetaboAnalyst Pathway analysis of HFD vs HFD-PHA.

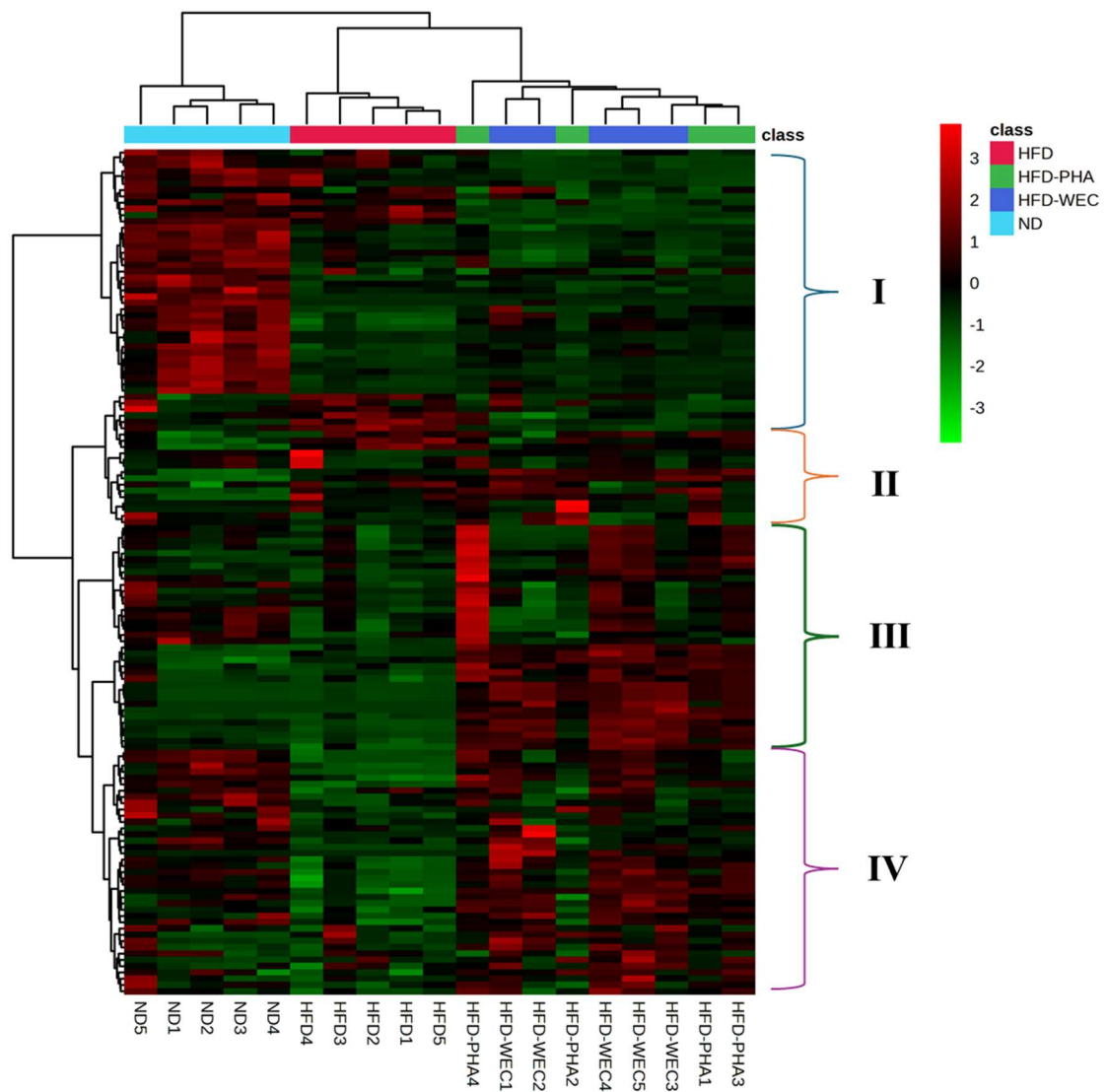
		Total Cmpd	Hits	Raw p	#NAME?	Holm adjust	FDR	Impact
1	Gluconeogenesis	30	8	2.64E-08	7.5779	2.06E-06	2.06E-06	0.22632
2	Glycolysis	20	6	1.19E-05	4.9262	0.00086521	0.000138	0.29455
3	Histidine Metabolism	35	2	1.81E-05	4.7428	0.0012474	0.000141	0.23416
4	Cardiolipin Biosynthesis	11	2	3.25E-05	4.4885	0.0021756	0.000204	0.2987
5	Glutathione Metabolism	19	2	4.86E-05	4.3132	0.0031112	0.000231	0.22903
6	Pentose Phosphate Pathway	27	7	6.61E-05	4.1798	0.0037737	0.000231	0.62952
7	Arginine and Proline Metabolism	48	7	8.84E-05	4.0537	0.0046339	0.000248	0.24651
8	Glycerolipid Metabolism	23	3	8.98E-05	4.0465	0.0046339	0.000248	0.22587
9	Fructose and Mannose Degradation	28	3	0.000112	3.949	0.0053831	0.000274	0.23464
10	Citric Acid Cycle	25	5	0.000164	3.7845	0.0072265	0.000366	0.20302
11	Nucleotide Sugars Metabolism	16	3	0.000182	3.7405	0.007815	0.000383	0.51724
12	Homocysteine Degradation	7	2	0.000348	3.4582	0.013231	0.000662	0.5
13	Inositol Phosphate Metabolism	22	2	0.000526	3.2786	0.018953	0.000933	0.22634
14	Urea Cycle	23	4	0.000724	3.1405	0.023837	0.001201	0.27951
15	Glycerol Phosphate Shuttle	8	1	0.002845	2.546	0.071115	0.004034	0.33333

Supplemental Table 6. MetaboAnalyst result joint-pathway HFD vs HFD-WEC.

	Total	Expected	Hits	Raw p	#NAME?	Holm adjust	FDR	Impact
Purine metabolism	231	0.91887	16	2.53E-16	15.597	8.28E-14	8.28E-14	0.22116
Pyrimidine metabolism	123	0.48927	7	5.01E-07	6.2998	0.00016345	8.20E-05	0.16981
Citrate cycle (TCA cycle)	52	0.20685	4	5.19E-05	4.2852	0.016802	0.004239	0.40605
Arginine biosynthesis	42	0.16707	3	0.000608	3.216	0.19461	0.023687	0.16092
Glycolysis or Gluconeogenesis	97	0.38585	3	0.006704	2.1737	1	0.11869	0.44994
Pentose phosphate pathway	67	0.26651	2	0.029075	1.5365	1	0.37784	0.22718

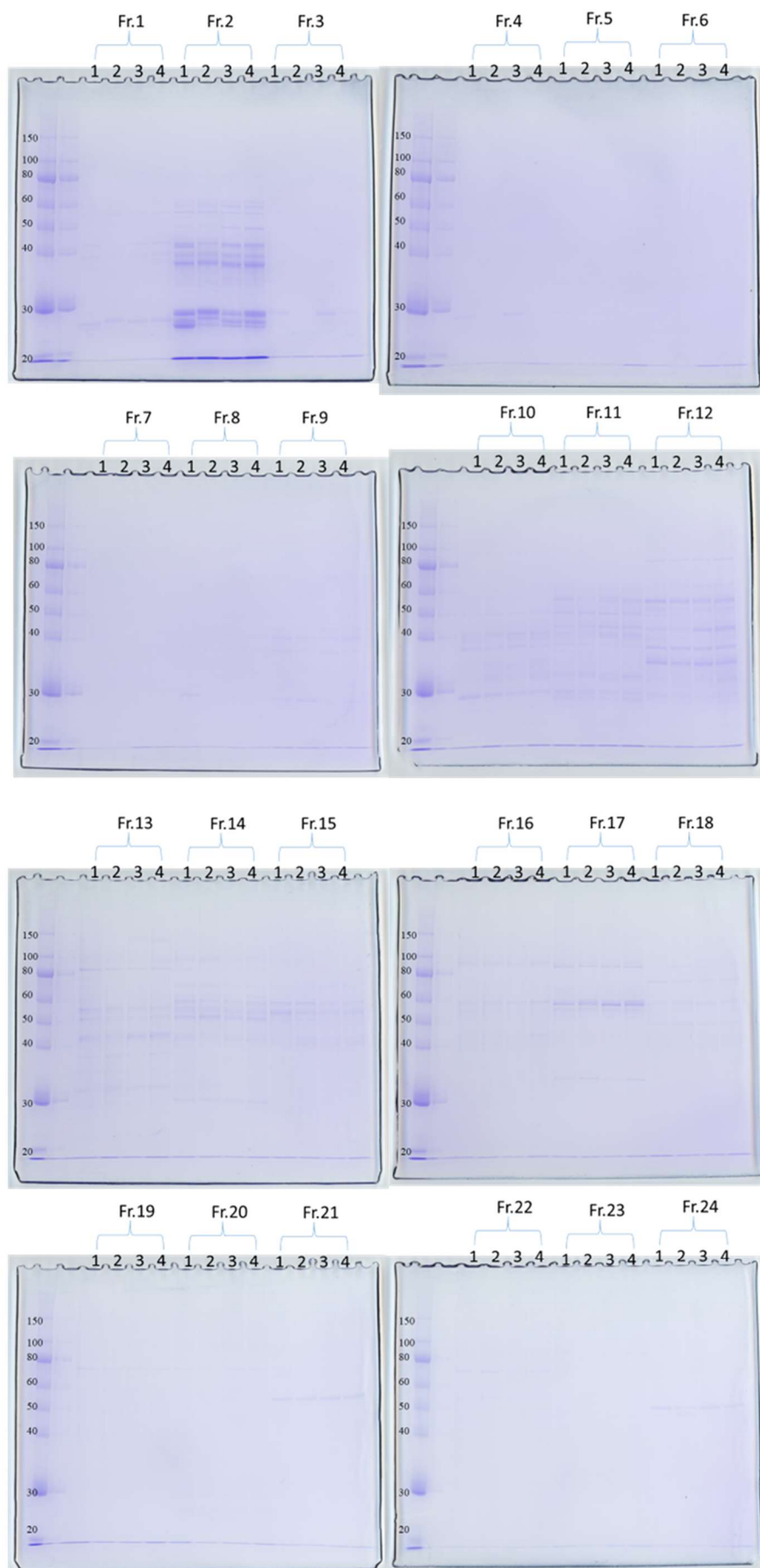
Supplemental Table 7. MetaboAnalyst result joint-pathway HFD vs HFD-PHA.

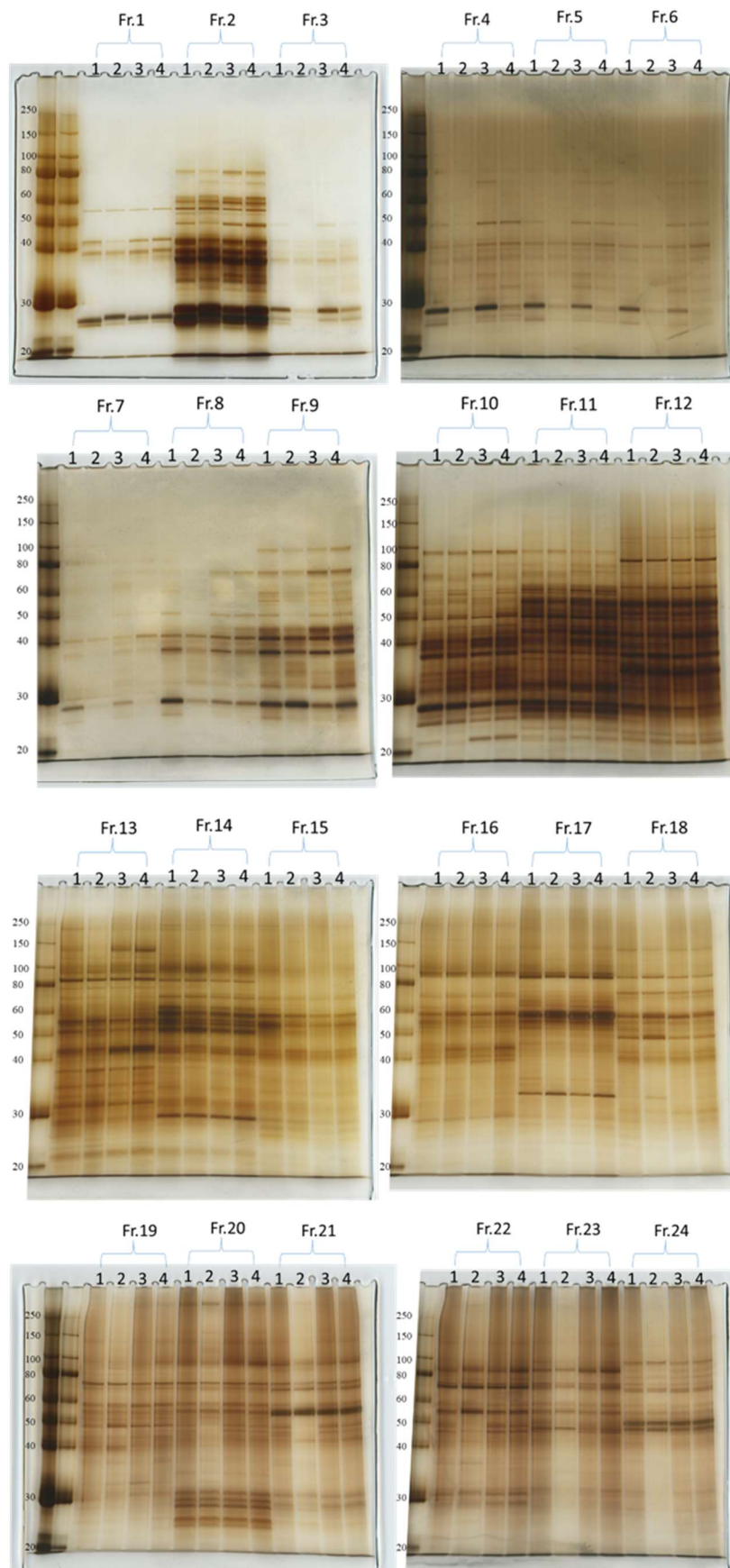
	Total	Expected	Hits	Raw p	#NAME?	Holm adjust	FDR	Impact
Glycolysis or Gluconeogenesis	97	0.38585	4	0.000582	3.2349	0.18398	0.015866	0.59393
Citrate cycle (TCA cycle)	52	0.20685	4	5.19E-05	4.2852	0.016698	0.002826	0.40605
Pentose phosphate pathway	67	0.26651	6	2.38E-07	6.6229	7.79E-05	7.79E-05	0.30854
Arginine biosynthesis	42	0.16707	4	2.21E-05	4.6561	0.0071522	0.001587	0.22059
Pyrimidine metabolism	123	0.48927	6	8.58E-06	5.0665	0.0027888	0.000935	0.11813
Cysteine and methionine metabolism	115	0.45745	4	0.001103	2.9576	0.34619	0.025752	0.089679
Glutathione metabolism	103	0.40971	3	0.007909	2.1019	1	0.13611	0.086364
Glycerolipid metabolism	99	0.3938	3	0.007092	2.1492	1	0.12884	0.038778



Supplemental Figure 1. Heatmap of the global 135 metabolites for all groups. Each row represents a metabolite, and each column represents a sample. The red color indicates up-regulation and green represents down-regulation.

A



B

Supplemental Figure 2. The gels were visualized with CBB (A) and silver stain (B).

Chapter 2 Comprehensive profiling of amino and carbonyl compounds in the Hong Yao sour soup, a type of fermented sour soup, in Huangluo Yao Village of Guangxi, China

Abstract:

Fermented sour soup is a traditional condiment in daily southwestern Chinese cuisine. Compounds found in Hong Yao sour soup were fractionated using size-exclusion chromatography. Amino compounds were identified through derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and liquid chromatography tandem mass-spectrometry (LC-MS/MS) with precursor scan, followed by identification using product ion scan. Other compounds lacking amino groups were isolated using solid-phase extraction with a strong cation exchanger and detected through LC-MS in full scan mode. Carbonyl compounds, including carboxylic acids, were detected and identified by derivatization with 2-nitrophenylhydrazine and LC-MS/MS. Besides amino acids and carboxylic acids including lactic and acetic acid, amino acid metabolites such as monoamines (tyramine, pyrrolidine, tryptamine), polyamines (cadaverine, putrescine), *N*-acetyl- and *N*-lactoyl-amino acids, and α -keto acids from Val, Ile, and Leu were also detected. However, unlike Japanese rice wine, only a few dipeptides were detected. This information aids in understanding the unique characteristics of Hong Yao sour soup.

1. Introduction

Fermented sour soup, a traditional condiment in southwestern Chinese cuisine, is divided into two types based on ingredients and fermentation methods. The first type,

red sour soup, is made from glutinous rice flour and vegetables such as tomatoes, red peppers, garlic, and ginger (Lin et al., 2020). An example of red sour soup is Kaili Hong Suan Tang. The second type, white sour soup, predates red sour soup and is primarily made from raw rice or glutinous rice flour without additional ingredients (Zhou et al., 2022b). Despite the differences, both types develop a sour taste after fermentation. A millennium ago, due to the humid climate and scarcity of salt, people in present-day Guizhou and Guangxi Province incorporated white sour soup into their diets as condiments (Wang et al., 2020). These fermented soups were believed to prevent illnesses like diarrhea and dysentery (Lin et al., 2022). Today, fermented sour soups are considered functional foods, reputed to aid digestion, boost appetite, and prevent obesity (Yuan et al., 2022; Zhou et al., 2022a). Moreover, fermented sour soup could be applied topically to prevent hair loss and maintain hair black.

Huangluo Yao Village, popularly known as "the long hair village," resides approximately 100 km from Guilin in the Longsheng Various Nationalities Autonomous County within northern Guangxi Zhuang Autonomous Region of China. This village is home to the Hong Yao ethnic group, among whom most women follow the tradition of maintaining long hair. The average hair length for adult Hong Yao women is 1.4 meters, with some reaching an impressive 2.3 meters. This earned them recognition from Guinness World Records as the "longest hair group." These women are renowned not only for their long hair but also for enduring black color even into their 80s. They attribute this hair care to their use of Hong Yao sour soup, a type of white sour soup, made from rice washing water at each home. A typical preparation method is as follows. The rice washing water is boiled on a stove before fermentation. After cooling, it is fermented at room temperature for a few days without using a starter. Fermentation is facilitated by airborne bacteria. In Huangluo Yao Village, this soup doubles as a condiment and a hair treatment.

In recent years, metabolomics techniques involving gas chromatography-mass spectrometry (GC-MS) have gained traction for analyzing bioactive and flavor components. Several studies utilizing GC-MS have revealed the presence of organic

acids, esters, alcohols, terpenoids, and amino acids in red sour soup (Wang et al., 2023). However, there is limited knowledge about the compounds within Hong Yao sour soup, which has been associated with maintaining black hair. Fermented products often contain an array of compounds, making their isolation and identification complex, even with GC-MS and LC-MS analyses. In previous research, LC-MS/MS was employed in precursor ion scanning mode along with derivatization techniques to identify compounds with distinct chemical groups in intricate matrices, such as Japanese fermented soy paste, miso (Shirako et al., 2020). This study aims to characterize amino and carboxyl compounds in Hong Yao sour soup using this approach to identify potential agents with hair-protecting properties.

2. Materials and methods

2.1. Sample

The Hong Yao sour soup was generously provided by Guilin Chang Fa Xiao Zhai Biotechnology Co., Ltd, Guangxi, China. Preparation method is outlined in the Supporting information (Supplementary Figure 1). The Hong Yao sour soup was subjected to centrifugation at 17,000 g for 10 min at 4°C. The resulting supernatant was stored at -30°C and utilized for subsequent experiments.

2.2. Reagents

Amino acids mixture standard solution (type H), triethylamine (TEA), phenyl isothiocyanate (PITC), n-butyric acid, isocaproic acid, propionic acid and sodium pyruvate were obtained from Wako Pure Chemical Industry (Osaka, Japan). 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ) was obtained from Toronto Research Chemicals (Toronto, Canada). Hydrochloric acid (6 mol/L), ammonium acetate, formic acid, 4-amino-n-butyric acid (GABA), ethanolamine, tyramine, phenylethylamine, cadaverine, histamine, putrescine, spermidine, spermine, acetic acid,

lactic acid, malic acid, n-caproic acid, succinic acid, heptanoic acid, acetonitrile (HPLC grade), methanol, and ethanol were purchased from Nacalai Tesque. (Kyoto, Japan). Glyceric acid, lithium acetoacetate, pyrrolidine, tryptamine, 4-methyl-2-oxovaleric acid, and *N*-acetyl-L-glutamic acid (*N*-Ac-Glu) were obtained from Tokyo Chemical Industry (Tokyo, Japan). Methionine sulfoxide, 2-ketobutyric acid, sodium 3-methyl-2-oxobutyrates, and 4-methyl-2-oxovaleric acid were purchased from Sigma Aldrich (St. Louis, MO, USA). The carboxylic acids assay kit, comprising 20 mM 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) in ethanol (reagent A) and 0.25 M 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) in ethanol/pyridine 97:3 (v/v) (reagent B), was purchased from YMC (Kyoto, Japan). Other chemicals used in this study were of analytical grade. Water purified through the Milli-Q system (Merck Millipore, Burlington, MA) was employed.

2.3. Amino acid analysis

Amino acid analysis was conducted using the percolumn PITC derivatization method with some modifications (Aito-Inoue et al., 2006). Glass tubes (50 mm × 6 mm i.d.) for hydrolysis were pretreated with 6 M HCl at 150°C for 1 h under vacuum. Following pretreatment, 20 µL of the sample were pipetted into the glass tubes and dried under vacuum. After drying, hydrolysis was performed using HCl vapor at 150°C under vacuum for 1h, followed by removal of HCl by vacuum. To each tube containing the hydrolysate or non-hydrolysate, 10 µL of an alkaline solution consisting of methanol, TEA, and water in a ratio of 7:2:1 was added and then dried. The dried sample was reacted with a 20 µL derivatizing solution consisting of methanol, TEA, water, and PITC in a ratio of 7:1:1:1 for 20 min at 25 °C. Excess reagent was removed by vacuum, and the residue was dissolved in 200 µL of 5 mM sodium phosphate buffer containing 10% (v/v) acetonitrile (pH 7.4). The solution was passed through a 0.45 µm filter (Merck Millipore). PITC derivatives were separated using a Shimadzu LC-20A high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) equipped with an L-column 3 C18 column (250 mm × 4.0 mm i.d., 5 µm; Chemicals

Evaluation and Research Institute, Tokyo, Japan) maintained at 45°C. Eluent A comprised 0.15 M ammonium acetate buffer, pH 6.0, containing 5% (v/v) acetonitrile, while eluent B was 100% acetonitrile. The gradient elution conditions were as follows: 0-5 min, 0% B; 5.0-5.1 min, 0-10% B; 5.1-25 min, 10-47.5% B; 25-30 min, 47.5-100% B; 30-37 min, 100% B; 37.1-40 min, 0% B. The injection volume was 20 µL, and the flow rate was 0.6 mL/min. Absorbance at 254 nm was monitored.

2.4. Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) analysis was performed using a Shimadzu LC-10A HPLC system. A Superdex Peptide 10/300 GL column (GE Healthcare, Piscataway, NJ) was utilized. Prior to separation, the sample was concentrated 5-fold under vacuum using a centrifuge concentrator. The injection volume was 200 µL. The eluent used was 0.1% formic acid, and the flow rate was set at 0.5 mL/min. Elution was monitored at 214 and 254 nm, and fractions were collected every 1 min.

2.5. Identification of compounds by LC-MS/MS

Amino compounds present in SEC fractions before the 45 minute mark were derivatized with AccQ and the resulting derivatives were subjected to analysis through LC-MS/MS in precursor ion scan mode, following the methods previously outlined by Boughton et al. (2011) and Ejima et al. (2018). Briefly, a 100 µL aliquot from each SEC fraction was dried using a vacuum centrifuge concentrator. The residue was dissolved in 10 µL of water and mixed with 20 µL of 0.3% AccQ acetonitrile solution and 70 µL of 200 mM sodium borate buffer (pH 8.8). This reaction mixture was then heated to 55°C for 10 min and subsequently filtered through a 0.45 µm filter. The filtrates were then analyzed using a Shimadzu LC-20A HPLC system coupled with a triple quadrupole mass spectrometer (LCMS-8040, Shimadzu) equipped with an electrospray ionization (ESI) source. Capillary voltage was set to 4.5 kV. Nitrogen drying gas and nebulizing gas flow rates were 15 L/min and 3 L/min, respectively. Collision-induced dissociation gas (Ar) pressure was 224 kPa. Heat block temperature and desolvation

line (DL) temperatures were set to 400 °C and 250 °C, respectively. Chromatographic separation was achieved on an Inertsil OSD-3 column (250 mm × 2.1 mm i.d., 5µm, GL Science, Tokyo, Japan) at a column temperature of 40°C, and the injection volume was 10 µL. A gradient LC system was operated using 0.1% formic acid in water (Eluent A) and 0.1% formic acid in water : acetonitrile at 20:80 (v/v) (Eluent B) at a flow of 200 µL/min. The gradient elution conditions were as follows: 0-15 min; 0-30% B, 15-20 min; 30-50% B, 20-25 min; 50-100% B, 25-30 min; 100% B, 30.1-40 min; 0% B. Specific detection of AccQ-derivatives involved selecting precursor ion that generated product ion of the AccQ-moiety (b1 ion, $m/z = 171.1$) in positive mode (collision energy of -35 eV) within the scan range of $m/z = 100-250$, $250-300$, $300-350$, $350-400$, and $400-500$. The precursor ions m/z values were recorded and subsequently analyzed using product ion scan mode (collision energy of -15, -25, and -35 eV) to estimate the structure of the amino compounds. Data processing was conducted using LabSolutions LCMS Ver. 5.5. (Shimadzu).

SEC fractions eluted after 45 min were also collected, as represented by peaks A-D in Figure 1. Aliquots (100 µL) from fractions A-D were vacuum-dried and then reconstituted in 30 µL of water for direct LC-MS analysis. The gradient elution conditions were as follows: 0-15 min; 0-30% B, 15-20min; 30-50%, 20-25min; 50-100% B, 25-30 min; 100% B, 30.1-40 min; 0% B. MS measurements were conducted in full scan mode (m/z 50-300), followed by product ion scan mode. Other LC-MS conditions remained consistent with those previously detailed.

For isolation of compounds lacking positive charge in the Hong Yao sour soup, a strong cation exchange spin column technique was employed, following a previously described protocol (Sato et al., 1998). The strong cation exchanger (AG 50W-×8 resin, hydrogen form, 100-200 mesh, Bio-Rad, Richmond, CA, USA) was packed into a Centrifugal Filter Device with a 5.0 µm pore size, (Ultrafree-MC, Merck Millipore, Burlington, MA, USA) and washed twice with 200 µL of 50% methanol, followed by four washes with water. After washing, 200 µL of the Hong Yao sour soup was pipetted onto the resin and elution was performed via centrifugation at 2000 g for 1 minute. The

unabsorbed fraction was directly subjected to analysis using the same LC-MS system. The gradient elution conditions were as follows: 0-30 min; 0-30% B, 30-40 min; 30-100% B, 40.1-50 min; 0% B. MS measurements were conducted in full scan mode (m/z 120-500), followed by product ion scan mode.

For carbonyl compounds within the Hong Yao soup, conversion to their 2-nitrophenylhydrazides was achieved using the carboxylic acids assay kit with slight modifications following the procedure outlined by Chen et al. (2019). A 50 μ L aliquot of the Hong Yao sour soup was combined with 50 μ L of reagent A and 50 μ L of reagent B in a 1.5 mL centrifugation tube, then incubated at 60°C for 20 min. After the reaction, the mixtures were cooled on ice for 1 minute before being filtered through a 0.45 μ m filter. For precursor ion scan of the derivatives in negative mode, the product ion with m/z 137 derived from 2-NPH was selected. The scan encompassed the range of m/z 150 to 800, with a collision energy of 15 eV. A 10 μ L aliquot was injected into the LC-MS/MS system and chromatographic separation was carried out on the Inertsil OSD-3 column at a column temperature of 45°C. The gradient LC system was operated using 0.1% formic acid in water (Eluent A) and 0.1% formic acid in acetonitrile (Eluent B), with the following gradient elution conditions: 0-1 min; 5% B, 1-40 min; 5-95% B, 40-45.5 min; 95% B, 45.6-55 min; 5% B. Other LC-MS conditions remained unchanged.

Detected compounds were identified by matching precursor and product ion m/z values with commercial standards, data in existing literature, and databases such as MassBank (<https://massbank.eu>) and Human Metabolome Database (HMDB, <https://hmdb.ca>).

2.6. Synthesis of pyroglutamyl peptide and N-lactoyl-amino acids

Pyroglutamyl peptides and *N*-lactoyl-amino acids standards were synthesized chemically from pyroglutamate or lactate and Fmoc-amino acids using a Shimadzu PSSM-8 solid-phase peptide synthesizer. The synthetic standards were purified using RP-HPLC, and their purity was confirmed by LC-MS following the methods detailed by Ejima et al. (2018). The content of the purified standards was determined through

amino acid release via HCl hydrolysis.

2.7. Quantification of compounds in Hong Yao sour soup

To quantify pyroglutamyl peptides, *N*-acetyl-, and *N*-lactoyl-amino acids, 10 μ L of samples or standards were directly subjected with varying concentrations (ranging from 0.01 μ M to 5 μ M) to LC-MS/MS in the multiple reaction monitoring (MRM) mode. Chromatographic separation was accomplished using the Inertsil OSD-3 column. A gradient LC system contains 0.1% formic acid (Eluent A) and 0.1% formic acid containing 80% acetonitrile (Eluent B). The gradient elution proceeded as follows: 0-10 min; 5-19% B, 10-27 min; 19% B, 27-40 min; 19-45% B, 40.01-46 min; 100% B, 46.01-55 min; 5% B.

For quantifying polyamines in the samples, the method from Fu et al. (2016) were followed with minor adjustments after derivatization with benzoyl chloride. Briefly, polyamine stock solutions (1 mg/mL) were prepared in purified water and stored at -30°C. Working standard solutions for calibration were prepared by diluting the stock solutions appropriately. To perform derivatization, 10 μ L of standard or sample (diluted one hundred times) was mixed with 45 μ L of 100 mM sodium borate buffer at pH 8.8 and 45 μ L of benzoyl chloride (2% in acetonitrile, v/v). The mixture was vortexed for 5 min at room temperature and then centrifuged at 13,000 *g* for 10 min at 4°C. The supernatant was transferred to the sample vial for LC-MS analysis. Derivatives were analyzed by LC-MS/MS in MRM mode using a Cosmosil 5C₁₈-MS-II column (150 mm $\times$ 2.0 mm i.d., 5 μ m, Nacalai Tesque). The column temperature was maintained at 40°C. A gradient LC system using 0.1% formic acid in water (Eluent A) and 0.1% formic acid containing 80% acetonitrile (Eluent B) at a flow rate of 200 μ L/min was employed. The gradient elution proceeded as follows: 2% B (0-1 min), 2-100% B (1-18 min), 100% B (18-22.5 min), followed by 2% B for 7 min to equilibrate the column.

For the quantification of monoamines, the sample was derivatized with AccQ, as described above. The derivatives were then analyzed using LC-MS/MS in MRM mode. Chromatographic separation was accomplished on a Cosmosil 5C₁₈-MS-II column. The

gradient elution conditions were as follows: 0-15 min; 0-30% B, 15-20min; 30-50% B, 20-25min; 50-100% B, 25-30 min; 100% B, 30.1-40 min; 0% B.

To quantify carbonyl compounds, the samples were converted to 2-nitrophenylhydrazide, following the method mentioned above with minor modifications. In short, 100 μ L of standard solutions or samples spiked with internal standard (IS) of heptanoic acid were mixed with 50 μ L of 200 mM 2-NPH $\cdot$ HCl in 50% aqueous methanol and 50 μ L of 120 mM EDC $\cdot$ HCl in pyridine/methanol (6:94, v/v). The mixtures were allowed to react at 40°C for 30 min. After the reaction, 800 μ L of 50% aqueous acetonitrile was added. The derivatives were analyzed using LC-MS/MS in MRM mode with chromatographic separation was achieved on the Inertsil OSD-3 column. A gradient LC system using 0.1% formic acid in water (Eluent A) and 0.1% formic acid in acetonitrile (Eluent B) at a flow rate of 200 μ L/min was employed. The gradient elution proceeded as follows: 0-1 min; 10% B, 1-11 min; 10-40% B, 11-35 min; 40% B, 35-45 min; 40-80% B, 45.1-51 min; 95% B; 51.1-60 min 10% B.

For each compound, MRM transitions were optimized using LabSolutions LCMS Ver. 5.5 by directly injecting 1 μ L of the individual derivatized standard solutions. Both commercially available standards and chemically synthesized standards were used for optimization. Each compound was monitored by three transitions and the most specific one was selected for quantification. Quantifier transitions are provided in Supplementary Table 1.

2.8. Statistical analyses

All statistical analyses were performed by using a GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Data were presented as mean $\pm$ SD. Differences in amino acid contents before and after acid hydrolysis were analyzed using the unpaired *t*-test for two independent means. A *p*-value < 0.05 was considered statistically significant.

3 Results

3.1. Amino acid contents

The amino acid contents of non-hydrolyzed and acid-hydrolyzed Hong Yao sour soup are presented in Table 1. In the current chromatographic conditions, GABA coeluted with Thr. As a result, the combined sum of GABA and Thr is shown in Table 1. Subsequent LC-MS/MS analysis revealed that the GABA content exceeded that of Thr. Consequently, the principal free amino acids in Hong Yao sour soup were GABA, Ala, Gly, Val, and Leu. Only minor quantities of Glu, Arg, Tyr, and Ser were present. Following HCl hydrolysis, a significant increase was observed in most amino acids ($p < 0.05$), except for GABA/Thr and Leu, indicating the presence of considerable peptide amounts. The rise in hydrophobic amino acids, such as Leu, Ile, Val, Ala, and Phe, due to the hydrolysis was less pronounced than that of hydrophilic amino acids.

3.2. SEC fractionations

Superdex Peptide 10/300 GL is designed to operate within a mass range of 20,000 to 100 Da. In practice, the optimal separation occurs in the range of 7,000 to 100 Da (Tran et al., 2011). Figure 1 illustrates the chromatograms of the fermented sour soup on Superdex Peptide 10/300 GL. Some peaks emerged between 27 and 42 min, aligning with the elution time of oligopeptides with 1,000 Da and water, respectively (Sato et al., 2013). Fractions were collected every minute from 27 to 44 min and labeled as SEC Fr. 28 to 45. Moreover, a series of broad and large peaks (A-D) appeared after the elution of water, persisting until 160 min. The compounds within peaks A-D were eluted subsequent to water, likely due to interactions with the column packing involving hydrophobic and/or electrostatic interactions. Peaks A-D were also collected.

3.3. Identification of compounds in SEC fractionations

Compounds in SEC Fr. 33 to 45 underwent derivatization with AccQ. This derivatization enhances the resolution and detection of hydrophilic compounds possessing primary and secondary amines through reversed-phase chromatography-mass spectrometry (Ejima et al., 2018). A precursor ion scan technique targeting the product ion of m/z 171 was employed for the specific detection of amino compounds. In SEC Fr. 33-35, only negligible peaks of amino compounds, aside from reagent peaks, were observed (data not shown). Figure 2A presents mass chromatograms of SEC Fr. 36-45, spanning scan ranges of m/z 100–250, 250–300, 300–350, 350–400, and 400–500. Peaks corresponding to amino acids were indicated using their one-letter abbreviations. Precursor ions within numbered peaks underwent product ion scan analysis. Peptide structure was deduced through immonium ions and sequence-specific ions—namely, a_n , b_n , x_n , and y_n ions, following the nomenclature in previous studies by Roepstorff and Fohlman (1984), and elaborated upon by Falick et al. (1993). This process resulted in the identification of 28 amino compounds, encompassing 6 dipeptides with Pro, Ala, and branched-chain amino acids, 3 monoamines (pyrrolidine, ethanolamine, and DOPA), 3 polyamines (cadaverine, putrescine, and *N*-acetyl spermidine), amino acids, and a modified amino acid (methionine sulfoxide). Table 2 provides a summary of retention time, precursor ion (m/z), product ions (m/z), and SEC fraction numbers for the estimated amino compounds. For compounds featuring a single amino/imino group, the AccQ derivative yielded a monovalent ion due to the presence of one AccQ moiety. Compounds with two amino/imino groups resulted in derivatives featuring two AccQ moieties, generating both monovalent and divalent ions. For instance, cadaverine and putrescine formed derivatives with 2 AccQ moieties, primarily generating divalent ions with m/z values of 222.2 and 215.2, respectively. However, a smaller portion of the putrescine derivative generated monovalent ions at m/z 429.3.

To detect the compounds within Peaks A-D, a total ion scan was executed using scanning ranges of m/z 50-120, 120-200, 200-250, and 250-300. Total ion

chromatograms (TIC) are depicted in Figure 2B. Solely monovalent ions, discerned through m/z differences between isotopes, were observed. Product ion scans were conducted for all precursor ions within numbered peaks to infer their structures. Table 2 also provides a summary of observed retention time, precursor ions (m/z), product ions (m/z), and estimated structures for Peaks A-D. Precursor and product ion patterns were cross-referenced with databases (HMDB and MassBank). Based on this information, tyramine, tryptophan, and L-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid were identified, as shown in Table 2. Notably, Supplementary Figure 2 showcases head-to-tail plots comparing the spectrum of m/z 217.1, obtained from product ion scan experiment at -15 V collision energy, with the corresponding in silico spectra predicted by HMDB. All identified compounds incorporate aromatic groups (phenyl and indole groups). Aside from the protonated molecular ion $[M+H]^+$ of tyramine at m/z 138.2, non-covalent homo-dimer ion, $[2M+H]^+$, was also detected at m/z 275.2.

3.4. Identification of compounds in cation exchanger non-absorbed fractionation

After separation through the cation exchange resin, compounds lacking positive charge, such as pyroglutamyl peptides, were eluted from the column. The eluates underwent analysis using LC-MS in the total scan mode. Figure 3 illustrates the TIC within scan ranges of m/z 120-200, 200-250, 250-300, and 300-500. The precursor ions within peaks marked with numbers were analyzed by LC-MS/MS in product ion scan mode. This analysis led to the identification of various compounds: organic acids, pantothenic acid, phosphorylated saccharides, pyroglutamyl peptides, and *N*-acetyl-amino acids. The summary of retention time, precursor and product ions, and estimated compounds is presented in Table 3. Conversely, precursor ions in peaks 9, 12, and 13 could not be definitively attributed to pyroglutamyl peptides, despite the presence of immonium ions corresponding to Gln ($m/z=84$) or Ile or Leu ($m/z=86$). The discrepancy between the full molecule masses and the estimated amino acid composition based on immonium

ions implies the existence of *N*-lactoyl-amino acids. These compounds are formed through dehydration condensation between the carboxy group of lactic acid and the amino group of amino acids. The estimated *N*-lactoyl (Lac)-amino acids, namely *N*-Lac-Gln, *N*-Lac-Leu, and *N*-Lac-Ile, were successfully synthesized. Confirmation of the presence of these *N*-lactoyl-amino acids was achieved by comparing their retention time and spectra with synthetic peptide standards via LC-MS/MS. For quantifying *N*-lactoyl-amino acids, LC-MS/MS with MRM detection was employed. The *N*-lactoyl-amino acid standards exhibited good linearity ($R^2 > 0.99$), ensuring dependable quantification. Notably, *N*-Lac-Gln registered at 1.43 μ M, followed by *N*-Lac-Leu (0.51 μ M), and *N*-Lac-Ile (0.34 μ M) (Table 4).

3.5. Carbonyl compounds

Carbonyl compounds, encompassing carboxylic acids, aldehydes, and ketones, can react with 2-NPH to form hydrazones (Hofstetter et al., 2019). Although the reaction mechanism differs between carboxyl group and ketone/aldehyde groups with 2-NPH, both derivatives yield a strong and stable product ion with m/z 137 in negative mode. This ion serves for the precursor ion scan in negative mode to detect carbonyl compounds in biological samples (Peters et al., 2004). Through the precursor ion scan, numerous carbonyl compounds were detected (Figure 4). The most prominent peak with m/z 224 was identified as lactic acid derivative through comparison with the standard. Similarly, the second most significant peak was identified as acetic acid with m/z 193 (Supplementary Figure 3A). Four minor peaks exhibited m/z values of 222, 236, 250, and 264 corresponding to molecular masses of 88, 102, 116, and 130, respectively, prior to derivatization. For identifying compounds with m/z 222 and 236, analogs of carbonyl compounds with these molecular masses were utilized as standards, including butyric acids (molecular weight of 88), pyruvic acid (88), 2-ketobutyric acid (102), and acetoacetic acid (102). By comparing the retention time and the product ions of the derivatives, compounds with molecular weights of 88 and 102 were identified as pyruvic acid and 2-ketobutyric acid, respectively (Supplementary Figure 3B and 3C).

Compounds with m/z 250 and 264 have molecular weights higher by 14 and 28 than 2-ketobutyric acid, indicating the presence of an additional methylene group. Based on comparisons with standards, peaks with m/z 250 and 264 were identified as 3-methyl-2-oxobutyric acid, 3-methyl-2-oxovaleric acid, and/or 4-methyl-2-oxovaleric acid (Supplementary Figure 3D). These α -keto acids have the potential to react with two 2-NPH molecules, although under the present conditions using 50 μ L of 20 mM NPH, only derivatives with one 2-NPH molecule were observed.

To achieve complete derivatives with two 2-NPH for quantifying these α -keto acids, derivatization was conducted using 200 mM 2-NPH in 50% methanol. LC-MS/MS revealed the presence of derivatives with two 2-NPH molecules. All derivatives exhibited doublet peaks corresponding to *cis* and *trans* isomers (Han et al., 2013) (Supplementary Figure 4). Given the limited separation of 3-methyl-2-oxovaleric acid and 4-methyl-2-oxovaleric acid under the current chromatographic conditions, the sum of these two compounds is reported in Table 4.

As mentioned earlier, a substantial peak of the 2-NPH derivative of lactic acid, a glucose metabolite, was identified. Consequently, other carboxylic acids from glucose metabolites and short-chain fatty acids commonly observed in fermented rice soup were quantified through 2-NPH derivatization and LC-MS/MS using MRM mode. The typical separation chromatograms obtained from a mixture of standards are shown in Supplementary Figure 5. Effective separations were achieved for ten 2-NPH derivatives. Calibration curves were constructed by plotting the peak area ratio of standards with varying concentrations to IS against the nominal concentration of each standard. Good linearity, indicated by high coefficients ($R^2 > 0.99$), and an extensive linear range were evident for all acids (Table 4). In Hong Yao sour soup, lactic acid exhibited the highest concentration (4761.2 mg/L), followed by acetic acid (616.3 mg/L). The remaining acids registered concentrations below 100 mg/L, with isocaproic acid not being detected.

3.6. Biogenic amines

LC-MS analysis of the SEC fractions indicated the presence of biogenic amines. The contents of 10 biogenic amines are listed in Table 4. All calibration curves demonstrated good linear regression ($R^2 > 0.99$). GABA stood out as the most abundant at 166.4 mg/L, followed by tyramine (44.1 mg/L) and putrescine (18.5 mg/L).

4. Discussion

The primary objective of this study was to characterize Hong Yao sour soup, a type of Chinese white sour soup believed to have benefits in preventing hair graying and promoting hair growth in elderly women. The main components of the sour soup, including amino acids, peptides, and carboxylic acids, were initially analyzed. Previous research has indicated the involvement of certain bacteria—such as lactic acid bacteria, acetic acid bacteria, and *Clostridium* spp. in the fermentation process of a similar white sour soup from another province, Guizhou (Zhou et al., 2022b). These bacteria are responsible for producing lactic acid, acetic acid, propionic acid, and butyric acid. This study also detected the presence of these carboxylic acids, confirming that the present preparation of Hong Yao sour soup exhibits the typical characteristics of Chinese white sour soup. The substantial amounts of lactic acid and acetic acid suggest that anaerobic fermentation by lactic and acetic acid bacteria occurs in Hong Yao sour soup. The presence of a smaller yet significant amount of butyric acid indicates the involvement of *Clostridium* spp. in the fermentation process. Due to the lack of pasteurization and the absence of starter use, variations in carboxylic acid levels may occur among producers, even though major carboxylic acids (lactic and acetic acids) exhibit similar levels. These carboxylic acids play a significant role as flavor compounds and preservatives in the sour soup. Additionally, aside from lactic acid and short-chain fatty acids, five α -keto acids were identified. Research has demonstrated that certain LAB species' aminotransferases can convert amino acids into corresponding α -keto acids (Gutsche et al., 2012). These findings indicate the presence of α -keto acids derived from

Thr, Ala, and branched-chain amino acids valine, leucine, and isoleucine in Hong Yao sour soup. These α -keto acids serve as precursors for other taste and flavor metabolites, such as aldehydes, alcohols, and carboxylic acids.

Free amino acids and peptides can contribute to taste of fermented condiments. Generally, peptides, particularly hydrophobic ones, tend to impart a bitter taste. Through HCl hydrolysis of soluble compounds in Hong Yao sour soup, hydrophilic amino acids, such as Asp, Glu, Gly, Ala, and Lys, were released predominantly, with only minimal amounts of hydrophobic amino acids such as Ile, Leu, and Phe being released. This observation indicates that the majority of peptides in the sour soup were composed of hydrophilic amino acids, while hydrophobic amino acids were present in their free form. This suggests the action of exopeptidases during fermentation, which preferentially release hydrophobic amino acids from peptides. As a result, the sour soup contains only small amounts of hydrophobic peptides, which contribute to a mild bitter taste and, consequently, does not possess a strong bitter flavor.

In previous studies, a solid phase extraction method were developed using the strong cation exchanger (AG 50W- $\times$ 8) packed into a mini spin column to isolate amino terminal blocked peptides. This method allowed the detection of pyroglutamyl peptides in various fermented foods. The present study also identifies the presence of pyroGlu-Leu. Pyroglutamyl peptides are shown to be formed non-enzymatically from peptides with glutaminyl residue at amino termina. Certain Japanese fermented foods such as Japanese rice wine (*sake*), soy paste (*miso*), and soy sauce (*shoyu*) contain relatively high levels of both hydrophilic and hydrophobic pyroglutamyl peptides, such as pyroGlu-Gln and pyroGlu-Leu, respectively (Shirako et al., 2019). In contrast, Hong Yao soup contained only a lower content (0.2 μ M) of pyroGlu-Leu compared to Japanese fermented products (40-100 μ M) (Kiyono et al., 2013). Furthermore, Hong Yao sour soup contained higher levels of *N*-acetyl- and *N*-lactoyl-amino acids compared to pyroglutamic peptides. Notably, *N*-acetylglutamate content exceeds that of free glutamate. In bacteria, *N*-acetyl-amino acids are synthesized from acetyl-CoA and amino acids through the enzyme *N*-acetylglutamate synthase (NAGS), which catalyzes

the production of *N*-acetylglutamate from glutamate and acetyl-CoA. The high content of *N*-acetylglutamate and the low content of pyroglutamyl peptide distinguish Hong Yao sour soup from Japanese rice wine, although both are derived from rice.

N-lactoyl-amino acids were first identified in Parmesan cheese. It has been reported that Korean fermented vegetable referred to *kimchi* and dry cured ham also contain *N*-lactoyl-amino acids (Christa et al., 2022; Paoletta et al., 2018). In *kimchi*, lactoyl-hydrophilic amino acids like Gly, Ala, Glu, Gln, and Asn were present. In contrast, Parmigiano-Reggiano cheese and ham contained lactoyl-hydrophobic amino acids such as Tyr, Met, Ile, Leu, and Phe. In Hong Yao sour soup, *N*-lactoyl-Gln, -Ile, and -Leu were found in amounts of 0.3-1.4 μ M, while free Gln was present at a lower level than other amino acids. These *N*-lactoyl-amino acids are reported to be enzymatically synthesized from lactic acid and amino acids during fermentation involving *L. helveticus* or *L. rhamnosus*, although the enzyme responsible for this synthesis in these bacteria has not been identified (Sgarbi et al., 2013). Jansen et al. (2015) identified an enzyme, cytosolic nonspecific dipeptidase 2 (CNDP2), in HEK 293 cells, that catalyzes the formation of *N*-lactoyl-amino acids. These findings suggest that *N*-lactoyl-amino acids are synthesized by exotype peptidases with certain substrate specificity, rather than by non-enzymatic reactions in Hong Yao sour soup. Studies have demonstrated that *N*-acetyl- and *N*-lactoyl-amino acids contribute to taste and taste modulation (kokumi perception) (Li et al., 2022; Zhao et al., 2016). However, the concentrations of these compounds in Hong Yao sour soup were considerably lower than the threshold concentrations for taste and kokumi perception.

Hong Yao sour soup contained monoamines and polyamines. Research has demonstrated that non-controlled autochthonous LAB or non-starter LAB are primarily responsible for the production of biogenic amine (BA) in fermented foods (Barbieri et al., 2019). The expression and/or activation of amino acid decarboxylation systems in LAB are considered adaptive responses to an acidic environment (Pereira et al., 2009). GABA, tyramine, and putrescine were the main BAs found in Hong Yao sour soup, with other amines also present. The content of GABA and tyramine exceeded that of

their precursor amino acids Glu and Tyr, respectively, suggesting significant decarboxylation, which may respond to accumulation of lactic acid and acetic acid. Extensive amino acid decarboxylation is a distinctive feature of Hong Yao sour soup.

Fermented sour soup serves not only as a culinary condiment but also as a traditional remedy for nurturing hair among Hong Yao women, who maintain long and black hair even in old age. It has been hypothesized that certain compounds in Hong Yao sour soup contribute to hair darkening and growth. However, there is currently no research data available regarding the active compounds responsible for promoting hair health in Hong Yao sour soup.

Hair graying is a common sign of aging in both animals and humans, regardless of gender. Numerous theories have been proposed to explain this phenomenon. Previous studies have suggested that the incomplete maintenance of melanocyte stem cells (MSCs) is a fundamental cause of hair graying (Nishimura et al., 2005). Another theory attributes hair graying to melanocyte apoptosis induced by oxidative stress (Arck et al., 2006). Hair loss is a more complex hair disorder influenced by aging, genetic predisposition, dietary habits, drugs, and hormonal and metabolic abnormalities (Hordinsky et al., 2002; Morinaga et al., 2021).

This present study highlights that Hong Yao sour soup contains common compounds such as amino acids and lactic acid. Additionally, it contains small quantities of specific compounds including short-chain fatty acids, peptides, BAs, *N*-lactoyl-amino acids, and *N*-acetyl-amino acids. Some of these compounds in Hong Yao sour soup may contribute to its hair-benefiting properties through antioxidant activity, protection, or activation of MSCs. Biogenic amines play essential roles in various cellular functions, including cellular proliferation and differentiation, nucleic acid regulation, protein synthesis, brain development, and nerve growth and regeneration (Shukla et al., 2010). In Hong Yao sour soup, the dominant BAs are GABA, tyramine, and putrescine. Recently, their potential role in hair follicle growth and pigmentation has garnered attention. Kovacevic et al. (2019) formulated a novel shampoo containing tyramine, which reduced hair shedding in women by contracting the *arrector pili*

muscle through the trace amine-associated receptor. Liu et al. (2002) reported that injecting additional spermidine into Merino lamb skin stimulated cell proliferation in the wool bulb and led to fiber growth. Ramot et al. (2010) reviewed evidence indicating that the polyamine spermidine stimulates melanin production in human anagen hair follicles cultured in vitro. Hong Yao sour soup contains all the aforementioned BAs, making them potential candidates as active compounds contributing to hair health among Hong Yao women. Additionally, other compounds like *N*-acetyl-amino acids, *N*-lactoyl-amino acids and short-chain fatty acids might also play a role in hair protection.

5. Conclusion

In this study, 63 compounds including amino acids, carboxylic acids, BAs, *N*-acetyl- and *N*-lactoyl-amino acids, and pyroglutamyl peptides were detected in Hong Yao sour soup. This study provides insights into the chemical constituents of Hong Yao sour soup. Based on existing literature, it can be assumed that hypothesize that BAs like tyramine and spermidine could contribute to hair protection. Ongoing research using animal models aims to explore the hair-protective activity of Hong Yao sour soup and its bioactive compounds. Therefore, the findings about the bioactive compounds in Hong Yao sour soup hold promise for both culinary and hair health perspectives.

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Legends for Figures

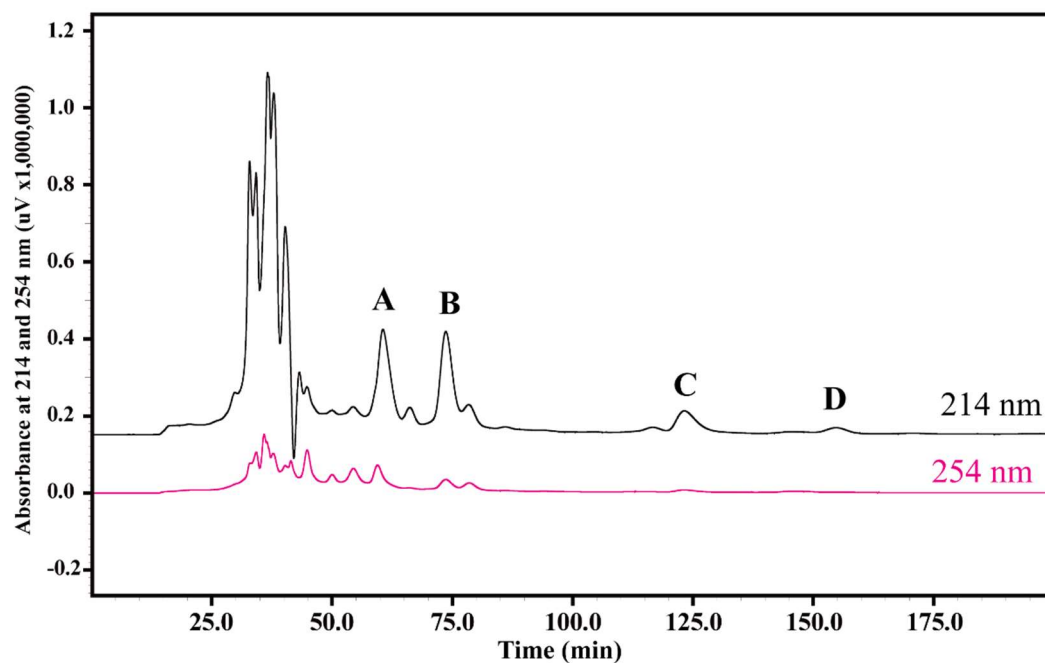


Figure 1. Size exclusion chromatogram of Hong Yao sour soup monitored at 214 and 254 nm. Fractions eluted from the Superdex Peptide 10/300 GL column were collected every 1 min from 27 min to 44 min. Four peaks eluted after 45 min were named A, B, C, and D, respectively.

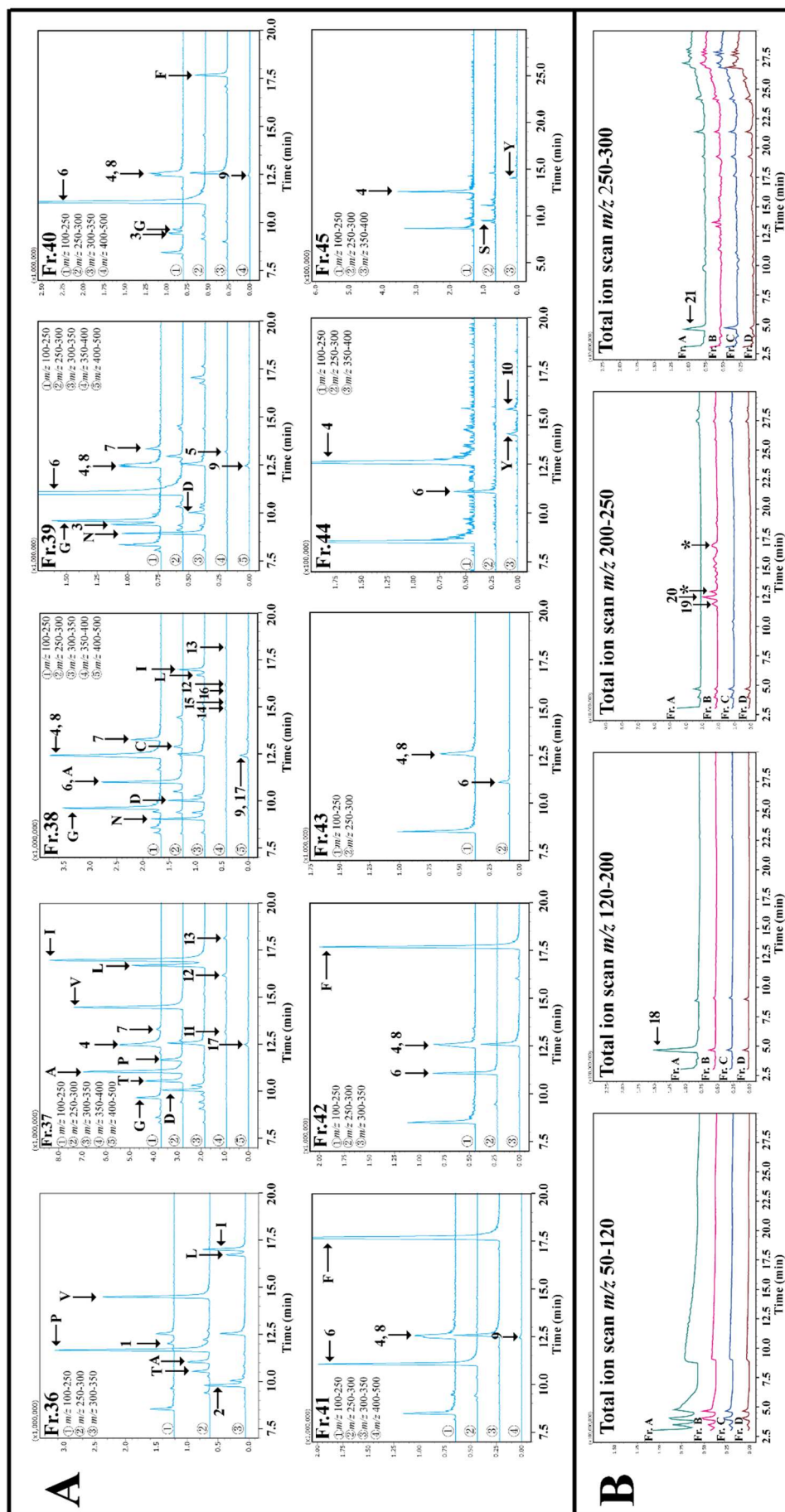


Figure 2. LC-MS chromatograms of SEC fractions of Hong Yao sour soup.

(A): LC-MS precursor ion scan chromatograms targeting m/z 171 of AccQ-derivatives in positive ion mode in SEC fractions 36-45. (B): LC-MS full scan chromatograms of non-AccQ derivatives in SEC fractions A-D with scanning ranges m/z 50-300. Compounds in peaks with numbers were further analyzed by product ion scan for estimation of structure. Peaks of proteinogenic amino acids were directly indicated with one-letter abbreviations of amino acids on peaks. Peaks that could not be detected in the product ion scan analysis are marked with an asterisk.

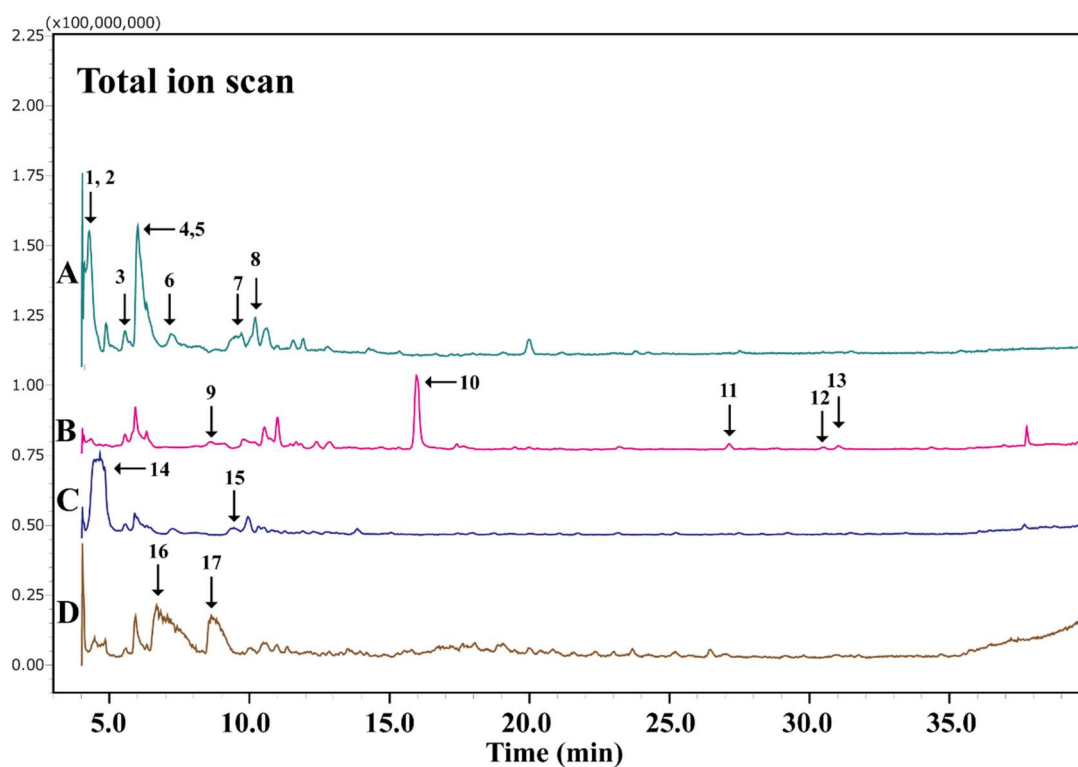


Figure 3. LC-MS full scan chromatograms obtained from the cation exchanger unabsorbed fraction at a scanning range of m/z 120-200 (A), 200-250 (B), 250-300 (C), and 300-500 (D) in positive ion mode. Compounds in peaks with numbers were further analyzed by product ion scan for estimation of structure.

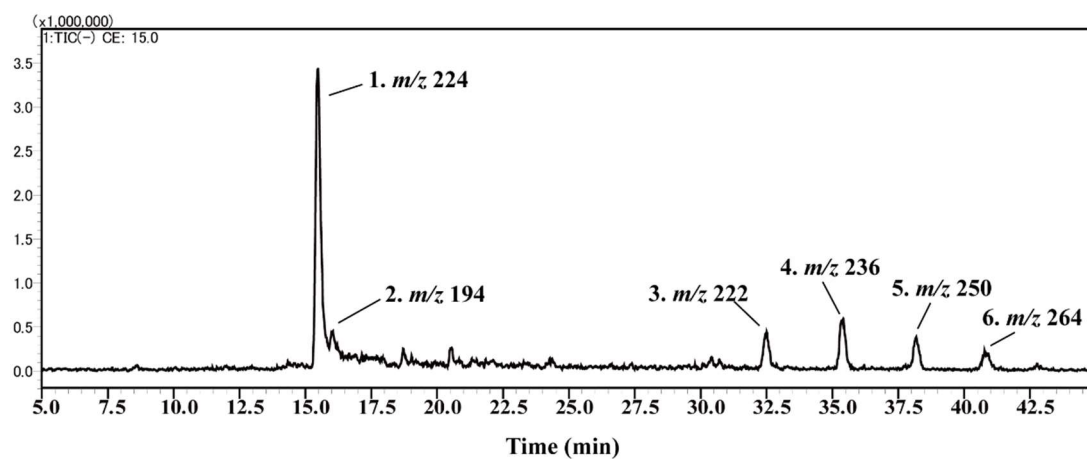


Figure 4. Precursor ion scan chromatograms targeting m/z 137 in negative ion mode of Hong Yao sour soup. Peaks identification: 1, lactic acid; 2, acetic acid; 3, pyruvic acid; 4, 2-ketobutyric acid; 5, 3-methyl-2-oxobutyric acid; 6, 3-methyl-2-oxovaleric acid and/or 4-methyl-2-oxovaleric acid.

Table 1. Concentrations of amino acids in Hong Yao sour soup and its HCl hydrolysate (mM).

Amino acids	non-hydrolysate	hydrolysate
Asp	0.53±0.02	1.26±0.05*
Glu	0.114±0.001	1.01±0.04*
Ser	0.051±0.003	0.59±0.05*
Gly	0.94±0.01	2.01±0.09*
His	0.20±0.01	0.36±0.02*
Arg	0.03±0.01	0.33±0.02*
Thr/GABA	2.35±0.02	2.47±0.10
Ala	1.20±0.01	1.82±0.07*
Pro	0.52±0.01	0.83±0.03*
Tyr	0.06±0.01	0.17±0.02*
Val	0.72±0.02	0.97±0.04*
Met	0.25±0.01	0.28±0.02*
Ile	0.306±0.001	0.45±0.02*
Leu	0.84±0.15	0.86±0.02
Phe	0.369±0.003	0.43±0.02*
Lys	0.34±0.01	1.08±0.03*
Asn	0.317±0.004	-
Gln	0.121±0.004	-

The data from triplicate analyses are given as mean ± SD. * $p < 0.05$ represent significant difference between non-hydrolysate and hydrolysate group.

Table 2. List of the identified compounds by LC-MS/MS analysis in the positive mode of AccQ-derivatives in SEC fractions 36-45 and non-AccQ derivatives in SEC fractions A and B from Hong Yao sour soup.

Peak No.	Retention time (min)	precursor ion (<i>m/z</i>)	Main product ions ^a (<i>m/z</i>)	Estimated compound	SEC Fr.
1	12.1	[242.1] ⁺	116, 130, 171	Pyrrolidine	36
2	10.8	[336.1] ⁺	56, 74, 100, 145, 166, 272	Methionine sulfoxide	36
3	9.6	[232.1] ⁺	116, 128, 143, 145, 171	Ethanolamine	39-40
4	12.5	[244.2] ²⁺	84, 116, 128, 145, 171, 172	Lysine	37-45
5	13.2	[368.1] ⁺	144, 171, 186, 214	Dopa	39
6	11.0	[274.1] ⁺	116, 128, 145, 171	GABA	38-44
7	13.5	[222.2] ²⁺	86, 116, 128, 145, 171, 172	Cadaverine	37-39
8	12.5	[215.2] ²⁺	72, 89, 116, 128, 145, 171	Putrescine	39-42
9	12.5	[429.3] ⁺	72, 98, 115, 145, 171, 285	Putrescine	38-41
10	15.4	[358.1] ⁺	171, 188, 358	N-Acetylspermidine	44
11	13.2	[357.2] ⁺	116(y1), 171, 187(y2), 242(b2)	Ala-Pro	37
12	16.2	[385.3] ⁺	72(V*), 116(y1), 145, 171, 215(y2), 270(b2)	Val-Pro	37-38
13	18.2	[399.2] ⁺	86(L*), 116(y1), 171, 229(y2), 256(a2), 284(b2)	Leu-Pro	37-38
14	15.0	[359.1] ⁺	72(V*), 145, 171, 189(y2), 242(a2), 270(b2)	Val-Ala	38
15	15.3	[373.2] ⁺	86(L*), 132.6, 145, 171, 203(y2), 256(a2), 284(b2)	Leu-Ala	38
16	16.2	[387.3] ⁺	72(V*), 145, 171, 217(y2), 242(a2), 270(b2)	Val-Val	38

Table 2. (continued)

Peak No.	Retention time (min)	precursor ion (m/z)	Main product ions ^a (m/z)	Estimated compound	SEC Fr.
17	12.5	[487.2] ⁺	84(K*), 130, 145, 147(y1)	Lysine	37-38
18	4.7	[138.2] ⁺	51, 65, 77, 91, 103	Tyramine	A
19	12.0	[205.1] ⁺	29, 91, 115, 118.2, 132, 146, 159, 170, 188	Tryptophan	B
20	12.7	[217.1] ⁺	106, 130, 144, 145, 146, 154, 156, 158, 171, 173, 184, 199	L-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid	B
21	4.7	[275.2] ⁺	77, 91, 93, 103, 121	2×Tyramine	A

^a Interpretation of a peptide CID spectrum relies mainly on the sequence-specific ions present (a, b, and c ion series and x, y, and z ion series and immonium ions (*)).

Table 3 Identified compounds by LC-MS/MS analysis of the cation exchanger unabsorbed fraction in positive mode from Hong Yao sour soup.

No.	Retention time (min)	precursor ion (<i>m/z</i>)	Main product ion (<i>m/z</i>)	Estimated compounds ^a
1	4.5	[197.0] ⁺	81,99	Ortophosphate
2	4.5	[140.1] ⁺	63,81,99	Ortophosphate+ACN
3	5.6	[148.1] ⁺	43, 60, 70, 88, 106, 130, 148	<i>N</i> -Ac-Ser
4	6	[132.1] ⁺	45, 91	Lactic acid+ACN
5	6	[181.1] ⁺	45, 91	2×Lactic acid
6	7.3	[127.1] ⁺	53, 63, 81, 99, 109	Ethylphosphate
7	9.5	[130.1] ⁺	28, 41, 56, 84	pyroGlu
8	10.2	[190.1] ⁺	43, 56, 84, 102, 130, 148, 172	<i>N</i> -Ac-Glu
9	8.8	[219.1] ⁺	56, 84, 90, 130, 184	<i>N</i> -Lac-Gln
10	16	[220.1] ⁺	30.5, 43, 72, 85, 90, 98, 124, 142, 184, 202	Pantothenic acid
11	27.0	[243.1] ⁺	43, 56, 84, 86(L*), 132(y1), 197, 226	pyrGlu-Leu
12	30.5	[204.1] ⁺	30, 41, 45, 69, 86(I*), 132(y1), 158	<i>N</i> -Lac-Ile
13	31.0	[204.1] ⁺	30, 44(L*), 45, 86(L*), 132(y1), 158	<i>N</i> -Lac-Leu
14	4.7	[261.0] ⁺	30, 43, 57, 81, 99, 109, 127, 145	Fructose 1-phosphate
15	9.5	[259.1] ⁺	84, 130	2×pyroGlu
16	7.0	[341.0] ⁺	43, 53, 81, 99, 109, 225, 243, 323	Fructose 1,6-bisphosphate

Table 3 (continued)

No.	Retention time (min)	precursor ion (m/z)	Main product ion (m/z)	Estimated compounds ^a	
17	9.0	[341.0] ⁺	43, 53, 81, 99, 109, 225, 243, 323	Glucose bisphosphate	1,6-

^a Abbreviations are: Lac-, Ac-, ACN: lactoyl-, acetyl-, acetonitrile, respectively.

Table 4 Contents of amino acid metabolites and carboxylic acids in Hong Yao sour soup.

Compound ^a	Concentration ^b	Calibration range	Linearity (r^2)	Units
<i>N</i> -Lac-Gln	1.43±0.01	0.2-5	0.9983	μM
<i>N</i> -Lac-Leu	0.51±0.03	0.01-2	0.9997	μM
<i>N</i> -Lac-Ile	0.34±0.03	0.01-2	0.9996	μM
<i>N</i> -Pyr-Leu	0.30±0.03	0.1-10	0.9993	μM
<i>N</i> -Ac-Ser	3.63±0.44	0.05-50	0.9980	μM
<i>N</i> -Ac-Glu	187.24±10.03 ^c	0.05-50	0.9998	μM
Pyruvic acid	0.52±0.03	0.005-0.5	0.9999	mg/L
2-Ketobutyric acid	0.017±0.002	0.0005-0.05	0.9998	mg/L
3-Methyl-2-oxobutyric acid	0.006±0.001	0.0005-0.05	0.9994	mg/L
3-Methyl-2-oxovaleric acid and 4-Methyl-2-oxovaleric acid	0.10±0.01	0.001-0.5	0.9999	mg/L
Succinic acid	87.91±8.18 ^c	0.001-5	0.9975	mg/L
Glyceric acid	1.68±0.15	0.005-5	0.9918	mg/L
Malic acid	0.027±0.003	0.001-2	0.9902	mg/L
Lactic acid	4761.21±115.28 ^c	0.5-10	0.9976	mg/L
n-Caproic acid	0.56±0.01	0.005-5	0.9982	mg/L
n-Butyric acid	0.05±0.01	0.05-5	0.9986	mg/L
Propionic acid	14.16±3.02 ^c	0.005-2	0.9988	mg/L
Acetic acid	616.33±63.20 ^c	0.05-5	0.9980	mg/L
Cadaverine	5.43±0.32 ^c	0.001-0.1	0.9994	mg/L
Putrescine	18.52±1.73 ^c	0.001-0.1	0.9991	mg/L
Spermidine	0.47±0.001 ^c	0.001-0.1	0.9987	mg/L
Spermine	0.87±0.03 ^c	0.001-0.1	0.9972	mg/L
Ethanolamine	8.01±0.14	0.05-10	0.9995	mg/L
Pyrrolidine	0.018±0.001	0.001-0.5	0.9985	mg/L

Table 4 (continued)

Compound ^a	Concentration ^b	Calibration range	Linearity (r^2)	Units
Methionine sulfoxide	1.77±0.08	0.05-5	0.9975	mg/L
GABA	166.39±10.00 ^c	0.05-5	0.9996	mg/L
Tyramine	44.09±2.09 ^c	0.005-0.5	0.9994	mg/L
Phenylethylamine	2.15±0.03	0.05-5	0.9995	mg/L
Tryptamine	7.59±0.58 ^c	0.005-0.5	0.9998	mg/L
Histamine	1.14±0.01	0.05-5	0.9999	mg/L

^a Abbreviations are: Lac-, Pyr-, Ac-: lactoyl-, pyroglutamyl-, acetyl-, respectively.

^b The data from triplicate analysis are given as mean ± SD.

^c Sample was diluted for quantification.

Supporting information

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Supplemental Table 1. Optimized mass spectrometric parameters for MRM analyses.

Analyte ¹	Precursor Ion (<i>m/z</i>)	Product Ion (<i>m/z</i>)	Q1 Pre- bias (V)	CE (V)	Q3 Pre- bias (V)
<i>N</i> -Lac-Gln	219.1	84.0	-18.0	-26.0	-17.0
<i>N</i> -Lac-Ile	204.1	158.1	-17.0	-11.0	-18.0
<i>N</i> -Lac-Leu	204.1	86.2	-17.0	-19.0	-17.0
<i>N</i> -Ac-Ser	148.2	60.1	-14.0	-18.0	-24.0
<i>N</i> -Ac-Glu	190.1	84.1	-16.0	-24.0	-18.0
<i>N</i> -Pyr-Leu	243.2	225.1	-12.0	-7.0	-17.0
Bz-Cadaverine	311.0	105.0	-13.0	-25.0	-20.0
Bz-Putrescine	297.0	105.0	-12.0	-26.0	-20.0
Bz-Spermidine	458.3	105.1	-12.0	-47.0	-21.0
Bz-Spermine	619.2	105.1	-26.0	-55.0	-12.0
Aqc-	232.0	170.7	-18.0	-17.0	-19.0
Ethanolamine					
Aqc-Methionine	336.0	170.9	-14.0	-25.0	-19.0
sulfoxide					
Aqc-GABA	274.0	171.0	-27.0	-20.0	-20.0
Aqc-Tyramine	308.2	171.0	-13.0	-23.0	-19.0
Aqc-	292.1	171.0	-24.0	-22.0	-19.0
Phenylethylamine					
Aqc-Tryptamine	331.1	171.0	-14.0	-30.0	-19.0
Aqc-Histamine	282.0	171.0	-24.0	-16.0	-19.0
Acetate-2NPH	193.9	122.1	19.0	14.0	21.0
Glycerate-2NPH	240.0	137.1	12.0	19.0	23.0
Lactate-2NPH	224.0	137.1	11.0	22.0	24.0
Malate-2NPH	403.0	208.2	11.0	19.0	20.0

Butyrate-2NPH	222.0	122.0	22.0	15.0	21.0
Caproate-2NPH	250.0	220.2	12.0	12.0	21.0
Isocaproate-2NPH	250.0	122.2	12.0	16.0	21.0
Propionate-2NPH	208.0	122.0	10.0	14.0	19.0
Succinate-2NPH	386.9	234.2	14.0	16.0	14.0
Pyruvate-2NPH	356.9	137.0	18.0	23.0	24.0
Alpha-Ketobutyrate-2NPH	370.9	137.1	18.0	23.0	24.0
2-keto-3-methylbutyrate-2NPH	384.9	137.0	28.0	25.0	24.0
3-Methyl-2-oxovaleric acid-2NPH	399.0	137.0	30	25	24
4-Methyl-2-oxovaleric acid-2NPH	399.0	137.0	21	26	24
Heptanoate-2NPH	264.0	234.2	12.0	12.0	15.0

¹Abbreviations are: Aqc, Bz-, Lac-, Pyr-, Ac-, 2NPH: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, benzoyl-, lactoyl-, pyroglutamyl-, acetyl-, 2-nitrophenylhydrazine, respectively.



Supplemental Figure 1. Preparation of Hong Yao sour soup in home and industry scale.

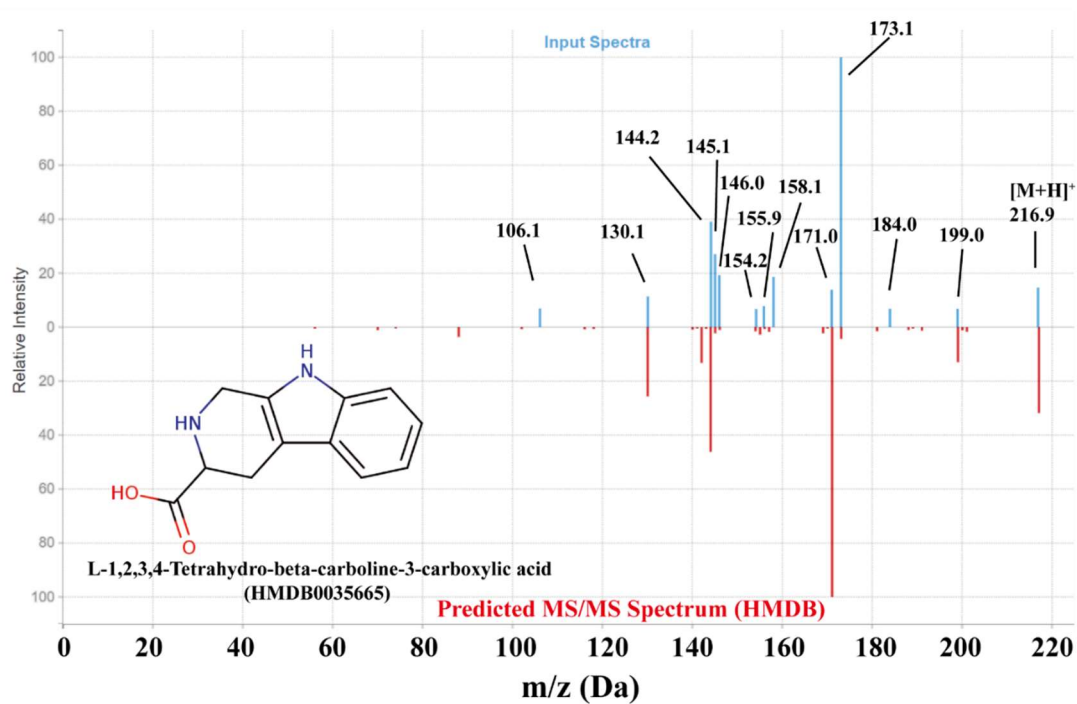
(A) Rice is harvested in Huangluo Yao Village.

(B) Rice is washed in water to get the rice washing water.

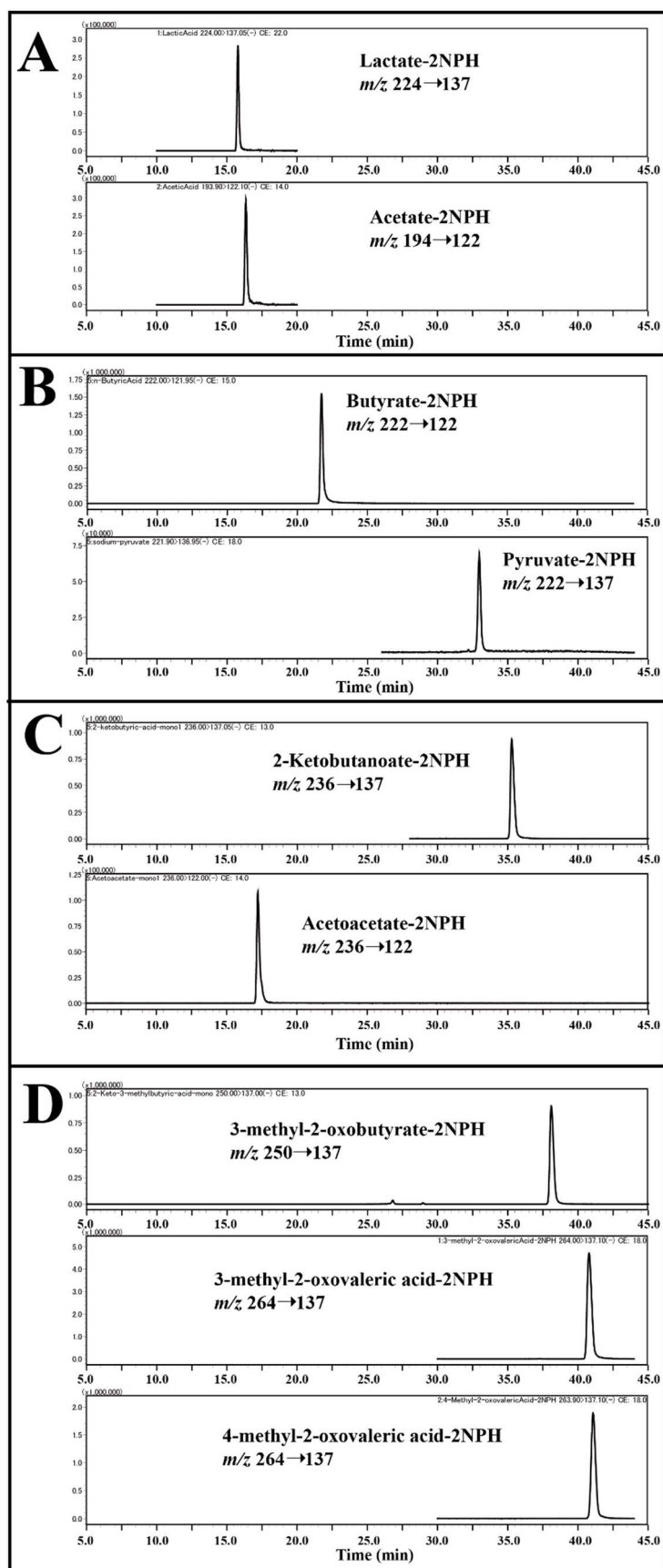
(C) After rice washing water is boiled on a stove in each home, it is fermented at room temperature for at least 7 days without using a starter. Fermentation is facilitated by airborne bacteria.

(D) Large-scale manufacturing is carried out by Guilin Chang Fa Xiao Zhai Biotechnology Co., Ltd located in Huangluo Yao Village. Local rice and water are used as raw materials. Homemade Hong Yao sour soup collected from several homes is used as the starter. During fermentation process, key quality parameters of pH and total acid number (TAN) are monitored for control of quality. The values are controlled within pH 2.5-3.0 and TAN > 3.0.

(E) The appearance of Hong Yao sour soup produced by this company, which was used in this study.



Supplemental Figure 2. Head-to-tail plot of experimental and predicted LC-MS/MS spectra of L-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid. The product ion scan was measured at -15 V, and the matching spectrum predicted by HMDB. The experimental input spectrum is shown on top in blue, and the closest matching result spectrum is shown underneath in red.



Supplemental Figure 3. LC-MS/MS chromatograms of the 2-NPH derivatized

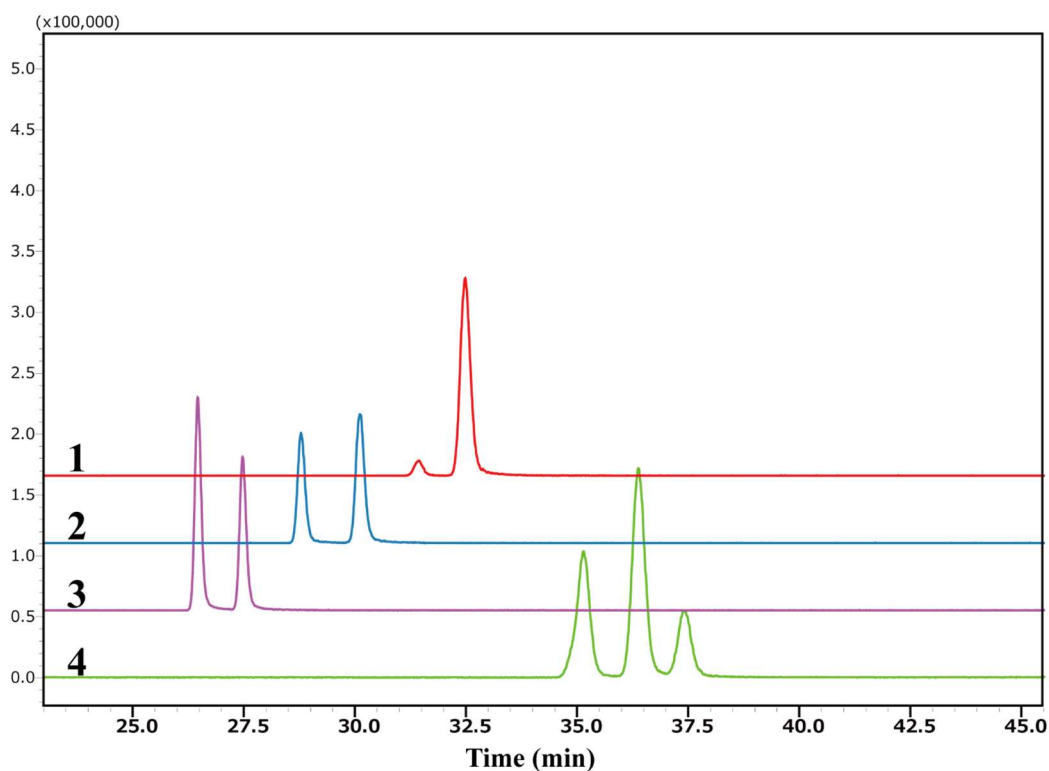
standards.

(A) MRM chromatograms of the 2-NPH derivatives of lactic acid and acetic acid.

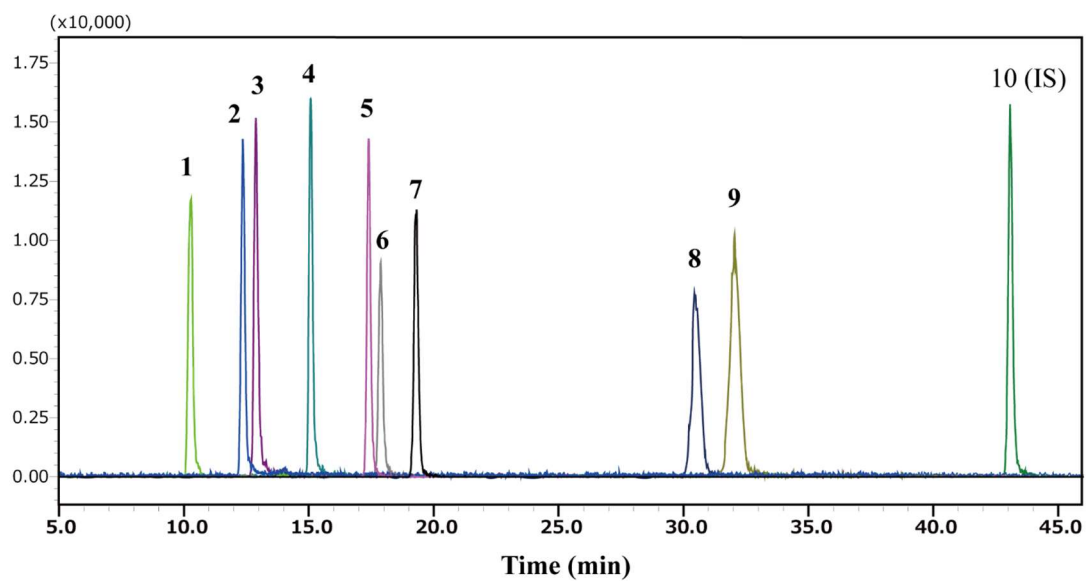
(B) MRM chromatograms of some 2-NPH derivatives of standard organic acids with precursor ion of m/z 222.

(C) MRM chromatograms of some 2-NPH derivatives of standard organic acids with precursor ion of m/z 236.

(D) MRM chromatograms of some 2-NPH derivatives of standard α -keto acids with precursor ion of m/z 250 and 264.



Supplemental Figure 4. The representative MRM LC-MS/MS chromatograms of the 2-NPH derivatives of a mixed standards of keto acids. Double peaks of *cis*(E)- and *trans*(Z)-stereoisomers were observed for all the derivatives. As 3-methyl-2-oxovaleric acid and 4-methyl-2-oxovaleric acid could not be separated completely under the present chromatographic conditions (Peak 4), sum of these compounds were present in Table 4. Peaks: 1, 3-methyl-2-oxobutyric acid; 2, 2-ketobutyric acid; 3, pyruvic acid; 4, 3-methyl-2-oxovaleric acid and 4-methyl-2-oxovaleric acid. Each peak corresponds to 500 ng/mL.



Supplemental Figure 5. The representative MRM LC-MS/MS chromatograms of the 2-NPH derivatives of a mixed standards. Peaks: 1, glyceric acid; 2, lactic acid; 3, acetic acid; 4, propionic acid; 5, malic acid; 6, n-butyric acid; 7, succinic acid; 8, isocaproic acid; 9, n-caproic acid; 10, heptanoic acid (internal standard). Each peak corresponds to 1000 ng/mL.

Chapter 3 Evaluation of the Efficacy of Topical Hong Yao Sour Soup and Biogenic Amine (BA) Complex on High Fat Diet-Induced Alopecia and Hair Pigmentation in Mice

Abstract:

Hong Yao sour soup (HYSS) has been traditionally used to promote hair growth and prevent graying in Huangluo Yao Village, Guangxi, China. To assess the effects of HYSS and its composition of trace amine complex (TAC) and poly amine complex (PAC) on HFD-induced alopecia and hair pigmentation, the fifteen-week-old C57BL/6J mice were fed either a normal diet (ND) or an HFD. HYSS, TAC, and PAC were topically applied to the hair-depilated skin of HFD-fed mice separately. Histological analysis showed that HFD feeding significantly suppressed hair growth compared to the ND group. Treatment with HYSS and TAC significantly increased hair follicle density in HFD-fed mice. Liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis revealed that HFD feeding led to hair depigmentation, which was reversed by only HYSS treatment. These findings indicate that the topical application of trace amines in HYSS may mitigate HFD-induced alopecia and show potential as a preventive and/or therapeutic agent for obesity-induced alopecia, while another compounds in HYSS are responsible for suppression of the hair depigmentation.

1. Introduction

Hair is a specialized structure derived from the skin, categorized as an accessory feature of the integumentary system alongside sebaceous glands, sweat glands, and nails (Park et al., 2011). Head hair plays several essential roles in both animals and humans, including providing protection from external impacts, aiding sensory perception,

facilitating perspiration evaporation, and regulating body temperature (Lee et al., 2020). Since hair is commonly viewed as a defining characteristic of individuals, significant attention is given to factors like hair quantity, thickness, and changes in color, such as graying. The prevalence of hair loss, or alopecia, continues to rise. In Japan, over 26% of men (approximately 13 million) experience hair thinning, and nearly one in ten women face similar issues (Sho et al., 2021).

Hair loss in both men and women is widely recognized as being influenced by genetic factors, hormonal imbalances, immune reactions, nutritional deficiencies, environmental factors, psychological stress, and aging (Liu et al., 2022). Additionally, several diseases, such as insulin resistance and obesity, are linked to hair loss. For example, a practice-based case-control study by Matilainen et al. (2000) suggested that insulin resistance may act as a pathophysiological mechanism or contributing factor to early-onset androgenetic alopecia (AGA). Obesity is a well-known risk factor for conditions like non-alcoholic fatty liver disease (NAFLD), type 2 diabetes (T2D), cardiovascular disease, cancer, and dyslipidemia. Morinaga et al., (2021) demonstrated that mice fed a high-fat diet (HFD) and subjected to repeated hair depilation exhibited severe and irreversible hair loss. Therefore, tailored treatments and care strategies are recommended for different types of alopecia.

Although hair loss is not a life-threatening condition, it can significantly impact an individual's self-esteem, mental health, and quality of life. Currently, only two medications—finasteride and minoxidil—are approved by the U.S. Food and Drug Administration for treating hair loss. Minoxidil, initially developed as an antihypertensive drug, was later found to promote hair growth when applied topically (Oh et al., 2014). Its mechanism of action involves opening adenosine triphosphate-sensitive potassium channels, dilating blood vessels, and enhancing the delivery of nutrients and oxygen to hair follicles (Meephansan et al., 2017). However, the effects of minoxidil are often temporary and vary among individuals, with adverse reactions like pruritus, scalp dermatitis, and local irritation being reported (Buhl et al., 1990; Messenger et al., 2004). Finasteride, a type II 5 α -reductase inhibitor, prevents the

conversion of testosterone to dihydrotestosterone (DHT), thereby reducing the binding of DHT to androgen receptors, which inhibits hair growth (Zhang et al., 2016). Despite its efficacy, finasteride is associated with several side effects, including sexual dysfunction, impaired muscle growth, and an increased risk of high-grade prostate cancer (Erdemir et al., 2008; Thompson et al., 2003). Moreover, due to its potential to cause male fetal malformation, it is contraindicated in pregnant or fertile women (Chung et al., 2017). Given the limitations of current treatments, the development of new therapeutic targets to enhance hair growth and prevent hair loss—beyond just AGA—is urgently needed in the hair care industry. Recent research has suggested that natural plant extracts and fermented products could serve as alternative or complementary therapies for hair loss, offering fewer side effects.

Trace amines (TAs), include tryptamine, tyramine, octopamine, and phenylethylamine (PHA), contain in certain foods, such as chocolate, aged cheese, and red wine (Branchek and Blackburn, 2003). Traditionally, the functional effects of TAs have been described as neuromodulation, meaning they can either increase or decrease a cell's response to different neurotransmitters (Zucchi et al., 2006). Their anti-obesity effects have also garnered substantial scientific interest in recent years (Brial et al., 2019; Lee et al., 2022). Moreover, Kovacevic et al., (2019) formulated a novel shampoo containing tyramine, which reduced hair shedding in women by contracting the arrector pili muscle through the trace amine-associated receptor (TAAR).

In Chapter 2, the Hong Yao women in Huangluo Yao Village popularly known as "the long hair village", are introduced. They are renowned not only for their long hair but also for enduring black color even into their 80s. They attribute this unique trait to their use of Hong Yao sour soup, a type of fermented rice water. In Chapter2, some key components of biogenic amines (BAs) and carboxylic acids were identified in the Hong Yao sour soup, revealing the presence of three trace amines (tyramine, PHA, and tryptamine) and four polyamines (cadaverine, putrescine, spermine, and spermidine), which may act as bioactive compounds contributing to the hair health of Hong Yao women.

To the best of my knowledge, few studies have explored the relationship between high-fat diet-induced alopecia and BAs. Therefore, the present study aims to investigate the effects of BAs on high-fat diet-induced alopecia in a C57BL/6 mouse model. Additionally, the influence of a high-fat diet on hair pigmentation was examined. The results may offer valuable insights for developing alternative therapeutic agents for patients suffering from obesity-induced organ dysfunction.

2. Materials and methods

2.1. Reagents

The Hong Yao sour soup (HYSS) was supplied by Guilin Chang Fa Xiao Zhai Biotechnology Co., Ltd, (Guangxi, China). Pyrrole-2,3,5-tricarboxylic acid (PTCA) was purchased from BLD Pharmatech Ltd. (Shanghai, China). Tyramine, PHA, cadaverine, putrescine, spermidine, spermine, lactic acid, 10%-formaldehyde neutral buffer solution, acetonitrile (HPLC grade), methanol, and ethanol were purchased from Nacalai Tesque (Kyoto, Japan). Tryptamine was obtained from Tokyo Chemical Industry (Tokyo, Japan).

2.2. Animal experiments

Fifteen-week-old male C57BL/6J mice, purchased from Japan SLC (Shizuoka, Japan), were used in this study. All the animals were treated and cared for according to the guidelines of the National Institutes of Health (NIH) for experimental animals. All experimental procedures were approved by the Animal Care Committee of the Louis Pasteur Center for Medical Research (Kyoto, Japan, approval number AR-2024-04-002). The animals were housed in polypropylene cages under standard conditions ($25 \pm 1^\circ\text{C}$, 40-80% humidity, and a 12-hour light/dark cycle). The mice had free access to water and food and were acclimated for one week prior to the study, which began when they were 16 weeks old. Before the experiments, hair in a 2×3 cm area on the back of

each mouse were removed using a depilatory cream (Veet, France), and depilation was repeated monthly.

Mice were randomly assigned to five groups (n=5 per group): 1. ND Group: Fed a standard diet (Certified Diet MF, Oriental Yeast, Osaka, Japan) without any treatments; 2. HFD Group: Fed an HFD (High-fat Diet 32, CLEA Japan, Tokyo, Japan), comprising 32% crude fat with 60% of the calories derived from the fat, without any treatments; 3. HFD-HYSS Group: Fed an HFD and treated topically with HYSS; 4. HFD-TAC Group: Fed an HFD and treated topically with a trace amine complex (TAC) (PHA, 2mg/L; tyramine, 44 mg/L; and tryptamine 8mg/L) in an aqueous lactate vehicle (5 mg/mL); 5. HFD-PAC Group: Fed an HFD and treated topically with a polyamine complex (PAC) (cadaverine, 5mg/L; putrescine, 19mg/L; spermidine 0.5mg/L; and spermine 1mg/L) in an aqueous vehicle. One day after depilation, mice were treated topically with 200 μ L of HYSS, TAC, or PAC using a pipette. The dose of amines in the treatments was calculated based on their concentration in HYSS, as detailed in Chapter 2. Topical treatments were administered five times per week for two months (Figure 1).

2.3. Histologic analysis and hair follicle density

At the end of the experiment, all mice were euthanized, and the skin from the treated areas was surgically removed using dissecting scissors. The samples were fixed in 10% formalin and embedded in paraffin blocks using standard procedures. Transverse sections of the skin were sliced into 4- μ m-thick fragments and stained with hematoxylin and eosin (H&E). Digital photomicrographs were taken of representative areas at 100 $\times$ magnification using an optical microscope (Eclipse Ci-L, Nikon, Tokyo, Japan). Hair follicles were manually counted in the dermis. The area of the dermis was measured using Image-Pro Plus 6.0 software (Media Cybernetics, Maryland, USA), with pixel data converted into millimeters (mm) using a conversion factor for the 100 $\times$ magnification. Hair follicle density was calculated using the following formula: Hair follicle density = Number of hair follicle (count) / Dermis area (mm²)

2.4. Quantitative analysis of eumelanin in hair by LC-MS

Approximately 1 mg of hair from each mouse was used for eumelanin assay. The hair was placed in a 2 ml Eppendorf tube, to which 450 μL of 1 M K_2CO_3 and 30 μL of 3.0% H_2O_2 were added. The mixture was heated in a boiling water bath for 20 minutes. After cooling, the residual H_2O_2 was decomposed by adding 50 μL of 10% NaHSO_3 , and the mixture was acidified with 250 μL of 6 M HCl . The resulting colorless to pale-yellow solution was extracted twice with 800 μL of ethyl acetate by vigorous mixing. The combined organic layers were evaporated to dryness in a vacuum concentrator, and the residue was dissolved in 1 mL of 80% acetonitrile and filtered through a 0.45- μm filter.

PTCA in the reactants was determined by LC-MS/MS in multi reaction monitoring (MRM) mode. Commercially available PTCA was used to optimize MRM condition. An aliquot of 2 μL of the sample was injected into the chromatograph. Chromatographic separation was performed using a Shodex HILICpak VN-50 2D (2.0 mm I.D. x 150 mm) column. Gradient elution was performed using a mobile phase of 5 mM ammonium formate solution containing 0.05% ammonium hydroxide (A) and acetonitrile (B) at a flow rate of 0.25 mL/min and a column temperature of 40°C. The gradient elution proceeded as follows: 0–1 min; 80% B, 1–9 min; 80–50% B, 9–14 min; 50% B, 14.01–25 min; 80% B.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 5.0; San Diego, CA, USA). Data are expressed as the mean $\pm$ standard deviation (SD). Comparisons between multiple groups were evaluated using one-way ANOVA followed by Dunnett's multiple comparison test. A p -value of < 0.05 was considered statistically significant.

3. Results

3.1. Gross hair growth and histologic analysis

To determine whether an HFD accelerates hair loss or thinning, aged mice with two induced hair cycles were fed either an HFD or a normal diet (ND) for two months. As shown in Figure 2a, by gross observation, HFD feeding significantly suppressed hair growth after the depilation compared to the ND group. Mice in the HFD group displayed fewer hairs and a visibly shaggy appearance. However, treatment with HYSS and TAC significantly rescued HFD-induced suppression of hair growth from the hair depilated skin.

To verify the gross morphology, transverse skin sections were analyzed to determine hair follicle density (Figure 2b). In ND-fed mice, the mean number of hair follicles was recorded at 100× magnification. The mean hair follicle density in the HFD group was 14.0 follicles, significantly lower than the 34.0 follicles observed in the ND group ($P < 0.05$). Mice treated with HYSS and TAC had mean follicle counts of 25.8 and 24.2, respectively, both of which were significantly higher than in the HFD group ($P < 0.05$). However, the hair follicle density in the PAC group was 17.2, which, although higher, was not significantly different from that in the HFD group.

3.2. Quantitative analysis of eumelanin in hair by LC-MS

The transition m/z 198.10→110.05 was selected as the MRM channel due to its maximum ion intensity and assay selectivity. The PTCA signal was reliably detected across a wide concentration range, providing excellent linearity. Calibration curves starting at 0.1 $\mu\text{g/mL}$ were linear up to 5 $\mu\text{g/mL}$, with high coefficients ($R^2 = 0.9955$), making the results sufficient for the study's objectives. The stationary phases containing diol functional groups in the Shodex VN-50 column provided better analyte retention compared to a reversed-phase (RP) column. For instance, as reported by Petzel-Witt and colleagues (2018), the retention time of PTCA on the Polaris C8-A column was

only 0.7 minutes, whereas, in this study, the retention time of PTCA was 4.8 minutes (Figure 3). As shown in Figure 4, normal hair samples exhibited a mean PTCA content of 1.6 $\mu\text{g}/\text{mg}$. However, after HFD feeding, the PTCA content was significantly lower, indicating hair decolorization. Treatment with HYSS reversed this decolorization, but treatment with PAC did not prevent hair fading in HFD-fed mice.

4. Discussion

The hair follicle (HF), an epithelial mini-organ, undergoes successive cycles: anagen (rapid growth phase), catagen (degeneration phase), and telogen (quiescence phase) (Müller-Röver et al., 2001). This cycling, which continues throughout an individual's life, relies on the presence of hair follicle stem cells (HFSCs) located in the bulge (Choi et al., 2021). During the anagen phase, HFSCs are activated by signals from the dermal papilla, which is adjacent to the bulge area (Hsu et al., 2011). Once activated, HFSCs exit the bulge, proliferating downward to produce differentiated progeny responsible for the cyclical regeneration of hair shafts (Greco et al., 2009; Morita et al., 2021). HFSC activity is significantly influenced by growth factors, hormones, and metabolic changes (Ozeki et al., 2003). It has been reported that HFD can drastically alter the HF's metabolic profile. Morinaga et al. (2021) reported that lipid droplets accumulated in HFSCs in mice after prolonged exposure to HFD. Oxidative damage, marked by increased 8-oxoguanine levels, was observed in HFSCs due to reactive oxygen species (ROS) generated during free fatty acid metabolism (Furukawa et al., 2004; Morinaga et al. 2021). Additionally, multiple inflammatory cytokines signaling pathways, including NF- κ B activation, were triggered in HFSCs of HFD-fed mice. This lipotoxicity and oxidative stress suppress Sonic Hedgehog (SHH) signaling in HFSCs, which is crucial for the development and cyclical regeneration of hair follicles, leading to premature anagen and a shortened hair cycle. Moreover, HFD-induced metabolic stress alters HFSC fate, ultimately leading to HFSC depletion. In agreement with these previously reported results, the present findings showed that HFD-fed older mice

(fifteen-week-old) exhibited severe and irreversible hair loss after two continuous months, with HFD feeding and two rounds of hair cycle induction. This model is therefore suitable for examining anti-obesity interventions and their effects on hair loss.

Despite extensive research into the transcriptional and epigenetic mechanisms that maintain HFSCs and their lineage differentiation, little is known about the metabolic pathways in HFSCs. Emerging evidence suggests that glycolytic metabolism plays a pivotal role in HFSC activity. Flores et al., (2017) demonstrated that several glycolytic metabolites, such as glucose-6-phosphate, fructose-bisphosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, and lactate, were consistently higher in HFSCs compared to the total epidermis. Increasing lactate production by blocking pyruvate entry into the TCA cycle significantly enhanced HFSC activation and initiated a new hair cycle, whereas lactate dehydrogenase (*Ldha*) deletion prevented the HFSC activation. A potential therapeutic approach for HFD-induced alopecia may involve reversing metabolic disorders, enhancing glycolytic metabolism, boosting antioxidant capacity, and promoting HFSC activation. In Chapter 1, the results showed that PHA, a type of TA, raised glycolysis intermediates, such as phosphoenolpyruvate, 2-phosphoglycerate, and lactate acid. PHA administration also significantly reduced HFD-induced lipid peroxidation. Also, the integrated metabolic and proteomic analysis suggested that supplementation with both WEC and PHA enhanced carbohydrate energy expenditure and liver antioxidation. In Chapter 2, the presence of TAs was examined, including tyramine, PHA, and tryptamine, in HYSS, a traditional Chinese hair treatment. Kovacevic et al., (2019) formulated a novel shampoo containing tyramine, which reduced hair shedding by contracting the arrector pili muscle via TAAR activation. The present findings align with these results, suggesting that trace amines may increase lactate acid production, activate HFSCs, and reduce HFD-induced alopecia through antioxidation.

Whether HFD treatment affected hair pigmentation in mice was also explored. Melanin is an irregular light-absorbing polymer containing indoles and other intermediate products derived from the oxidation of tyrosine by tyrosinase in

melanocytes (Hearing et al., 1999). Melanocytes produce two major chemically distinct types of melanin pigments: brown-black eumelanin and red-yellow pheomelanin (Parsad et al., 2003). Due to eumelanin's relative insolubility in both acidic and alkaline solutions, its poorly defined physio-chemical properties, strong binding affinity to protein components, and susceptibility to structural alterations through radical isolation procedures, directly quantifying melanin has proven challenging. Most attempts have been met with limited success (Szekely-Klepser et al., 2005). One common approach involves quantifying the degradation products of eumelanin in biological samples after alkaline H_2O_2 oxidation or acidic KMnO_4 oxidation (Ito et al., 1998). These methods have gained popularity among researchers since they allow for the estimation of melanin content without requiring isolation. The methods are based on the formation of specific degradation products, such as PTCA, which is a quantitatively significant indicator of eumelanin. However, these methods present some disadvantages: first, chromatographic separation is typically performed on a reverse-phase HPLC column, which is not ideal for the hydrophilic PTCA. Second, the UV absorption-based detection of PTCA has relatively high detection limits.

To overcome these limitations, a modified method has been developed for quantitatively analyzing eumelanin in biological samples using LC/MS/MS. This assay involves a simple oxidation of eumelanin in mouse hair with K_2CO_3 and H_2O_2 , followed by hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-LC-MS/MS) analysis in MRM mode with electrospray ionization. This technique measures PTCA concentrations without requiring further derivatization. The advantages of HILIC-LC-MS/MS include its ability to retain PTCA, which would elute too quickly in RP chromatography. Additionally, detection in alkaline conditions and negative ion mode promotes deprotonation of carboxyl groups during ionization, significantly improving the signal-to-noise ratio for the pseudo-molecular ion.

Interestingly, hair decolorization was observed in mice following treatment with HFD. Previous studies have suggested that the incomplete maintenance of melanocyte stem cells (MSCs) in the hair follicle niche is a fundamental cause of hair graying in

both mice and humans, which results in loss of melanogenically active progeny (melanocytes) in the hair matrix (Nishimura et al., 2005). Based on these findings, the present author hypothesizes that HFD-induced stress not only targets HFSCs, accelerating hair thinning, but may also target MSCs, leading to hair decolorization. However, the specific mechanisms underlying these effects require further investigation.

According to Ramot et al., (2010) the polyamine, spermidine, stimulates melanin production in human anagen hair follicles cultured in vitro. In Chapter 2, the presence of polyamines, including cadaverine, putrescine, spermidine, and spermine in HYSS was shown. To test whether HYSS polyamines or TAs could reverse hair decolorization, further experiments were conducted. Unfortunately, aside from HYSS, neither TAs nor polyamines were effective in reversing this fading.

5. Conclusions

This study highlights the potential of HYSS and TAs in preventing HFD-induced damage and improving hair pigmentation as part of anti-alopecia therapies. At least monoamines in HYSS contribute to future investigations should focus on understanding the specific effects of TAs on HFD-induced alopecia and identifying the compounds in HYSS responsible for enhancing pigmentation.

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Legends for Figures

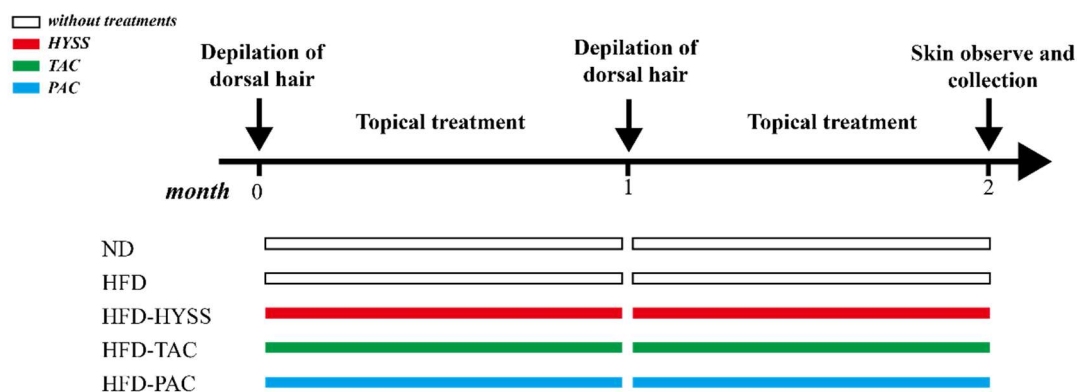


Figure 1. Experimental scheme. Dorsal hairs of 16-week-old C57BL/6J mice were depilated, and depilation was repeated monthly. The mice were divided into following 5 groups: 1. ND Group: Fed a standard diet; 2. HFD Group: Fed an high-fat diet (HFD); 3. HFD-HYSS Group: Fed an HFD and treated topically with Hong Yao sour soup (HYSS); 4. HFD-TAC Group: Fed an HFD and treated topically with a trace amine complex (TAC) in an aqueous lactate vehicle (5 mg/mL); 5. HFD-PAC Group: Fed an HFD and treated topically with a polyamine complex (PAC) in an aqueous vehicle. One day after depilation, mice were treated topically with HYSS, TAC, or PAC. Topical treatments were administered five times per week for two months. Depilated dorsal skin samples were collected in the 2nd month after depilation and stained with hematoxylin and eosin.

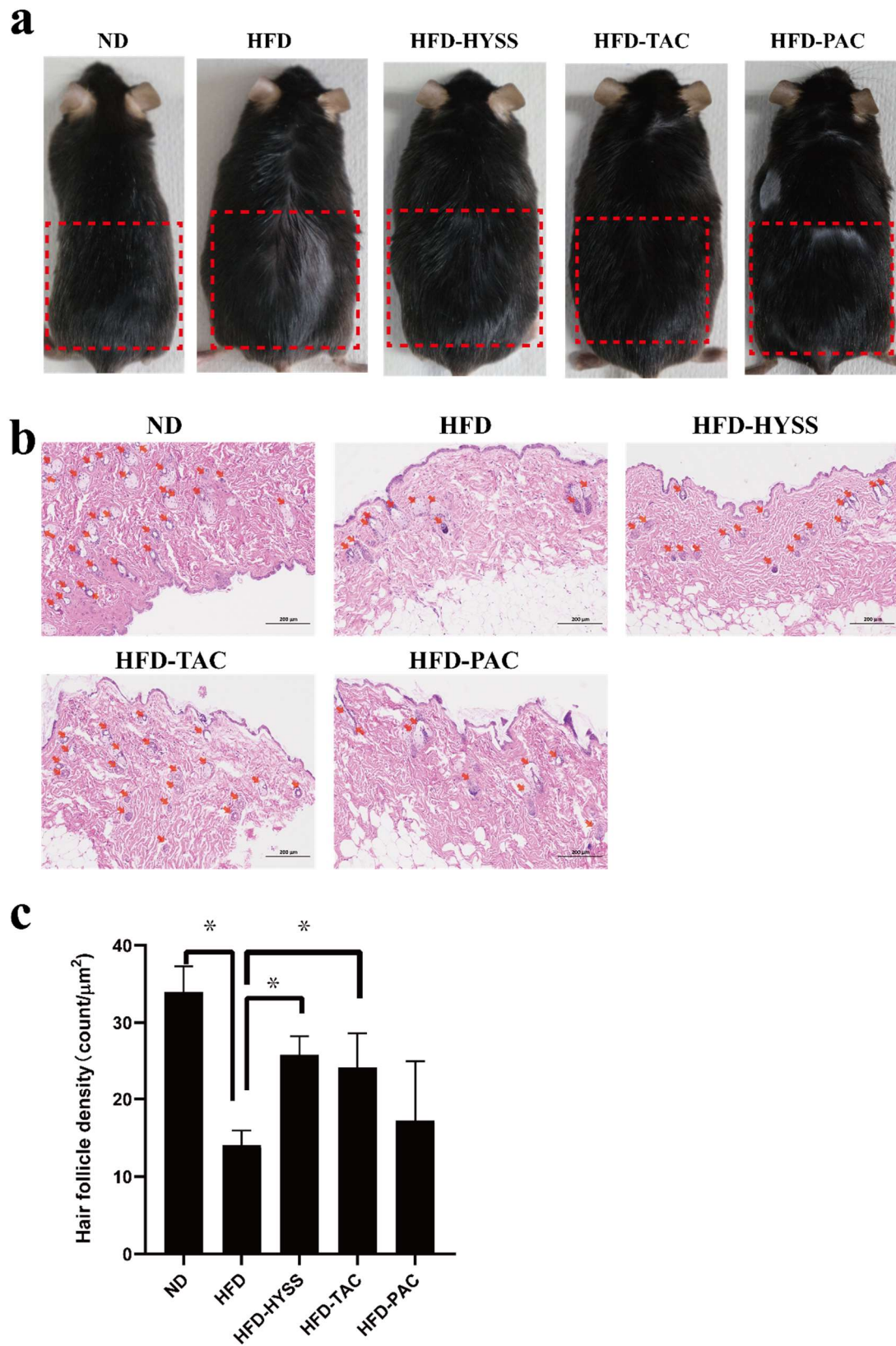


Figure 2. Topically treatment with trace amines in HYSS partially rescues HFD-induced hair thinning. a. Representative images of macroscopic evaluation on hair

growth; b. Hematoxylin–Eosin (magnification $\times$ 100, scale bar 200 μ m) staining of the skin transverse sections. Hair follicles are represented by arrows; c Quantitative analysis on the hair follicle density. Values represent the mean $\pm$ standard deviation. *P < 0.05, compared to HFD group.

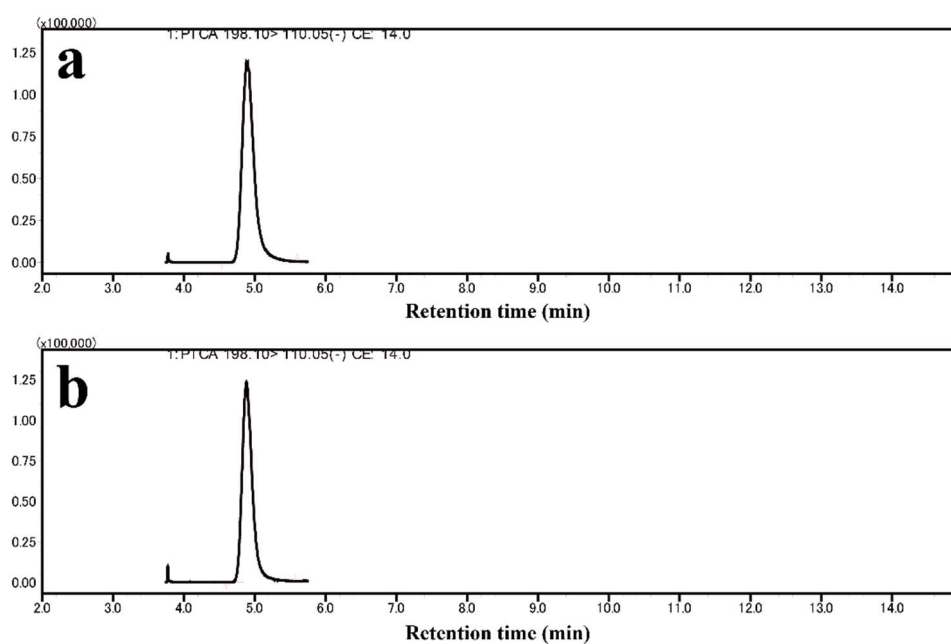


Figure 3. The representative MRM- LC-MS/MS chromatograms of the PTCA standards and sample. Peak a corresponds to 2.5 $\mu\text{g/mL}$ and peak b corresponds to a sample of ND group.

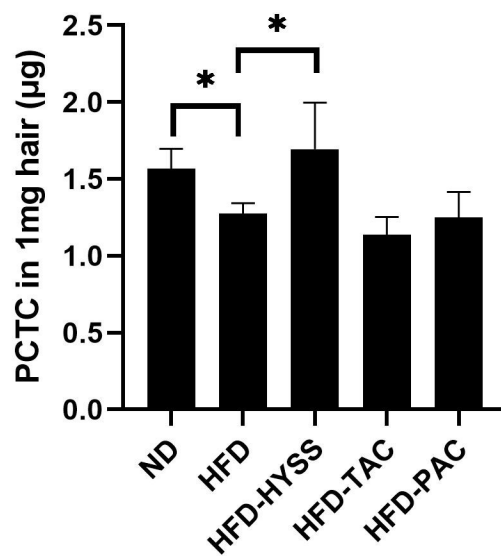


Figure 4. PTCA concentrations by LC-MS/MS in 5 groups hair samples. Values represent the mean $\pm$ standard deviation. * $P < 0.05$, compared to HFD group.

Total conclusion

This study provides compelling evidence for the beneficial effects of trace amines (TAs), particularly phenylethylamine (PHA), in mitigating metabolic dysfunction and hair thinning in high-fat diet (HFD)-induced obese mice. Through targeted metabolomic and proteomic analyses, the present results demonstrated that supplementation with chlorella water extract (WEC) and PHA led to significant alterations in key metabolic pathways, including glycolysis, gluconeogenesis, the citrate cycle, and arginine biosynthesis. These changes contributed to improved glucose metabolism, reduced oxidative stress, and enhanced detoxification processes in the liver.

The present results further revealed that the administration of PHA and WEC not only modulated the expression of proteins involved in energy metabolism and antioxidant defense but also corrected disruptions in the urea cycle, which plays a critical role in ammonia detoxification. These findings suggest that PHA and WEC supplementation can restore metabolic balance in obese mice, potentially offering a therapeutic approach for managing non-alcoholic fatty liver disease (NAFLD) and other obesity-related disorders.

Additionally, the present investigation into the application of Hong Yao Sour Soup, containing TAs, demonstrated its efficacy in promoting hair growth and pigmentation in HFD-induced hair-thinning mice. This highlights the potential of TAs not only in metabolic regulation but also in addressing cosmetic concerns related to obesity.

In conclusion, the dual impact of TAs in foods including chlorella and some fermented foods on both metabolic health and hair restoration presents a novel approach to the treatment of obesity-related conditions. Future studies should focus on understanding the precise molecular mechanisms of TAs in these processes and exploring their therapeutic applications in clinical settings. The use of natural bioactive compounds such as TAs could pave the way for safer, more effective treatments for obesity and its associated complications.

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