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Metabolomics study on fermented soybean meal by *Rhizopus oligosporus* RT-3 and its improvement on the growth status of malnourished mice

Teng Sun[1], Zhaojun Wang[1], Yiqiang Dai[1], Kang Zhai[1], Xiangna Fang[1], Yitian Chen[1], Baomin Zhao[2] and Mingsheng Dong[1*]

Abstract

In this study, *Rhizopus oligosporus* RT-3 was utilized to ferment soybean meal to obtain a tempe-like food with low fat and high bioactivity. To investigate the impact of fermentation on soybean meal's nutritional value, we detected the protein-related indices and conducted a non-targeted metabonomic analysis on both fermented soybean meal (FSM) and soybean meal (SM) in order to explore changes in a certain type of metabolite and its corresponding metabolic pathways. The results showed a significant increase in crude protein, peptides and amino acids while getting porous after fermentation. A reduction of raffinose and stachyose to 18.1% and 8.5%, respectively, due to the action of α-galactosidase and β-furanofructosidase occurred. As to amino acid metabolites, essential amino acids for human bodily functions such as lysine, histidine, and threonine were significantly upregulated. Furthermore, nine peptide metabolites were identified as angiotensin-converting enzyme (ACE) inhibitors and dipeptidyl peptidase-4 inhibitors (DPP-IV). After feeding with different doses of FSM, levels of insulin-like growth factor-1 (IGF-1), hemoglobin (HB), albumin (ALB), and total protein (TP) of malnourished mice all returned to normal. In conclusion, fermentation with *Rhizopus oligosporus* RT-3 can improve the quality of SM by reducing the level of oligosaccharide anti-nutrition factors and increasing the contents of free amino acid and small bioactive peptides, which provide a theoretical basis and new ideas for the application of FSM.

*Correspondence:
Mingsheng Dong
dongms@njau.edu.cn
Full list of author information is available at the end of the article

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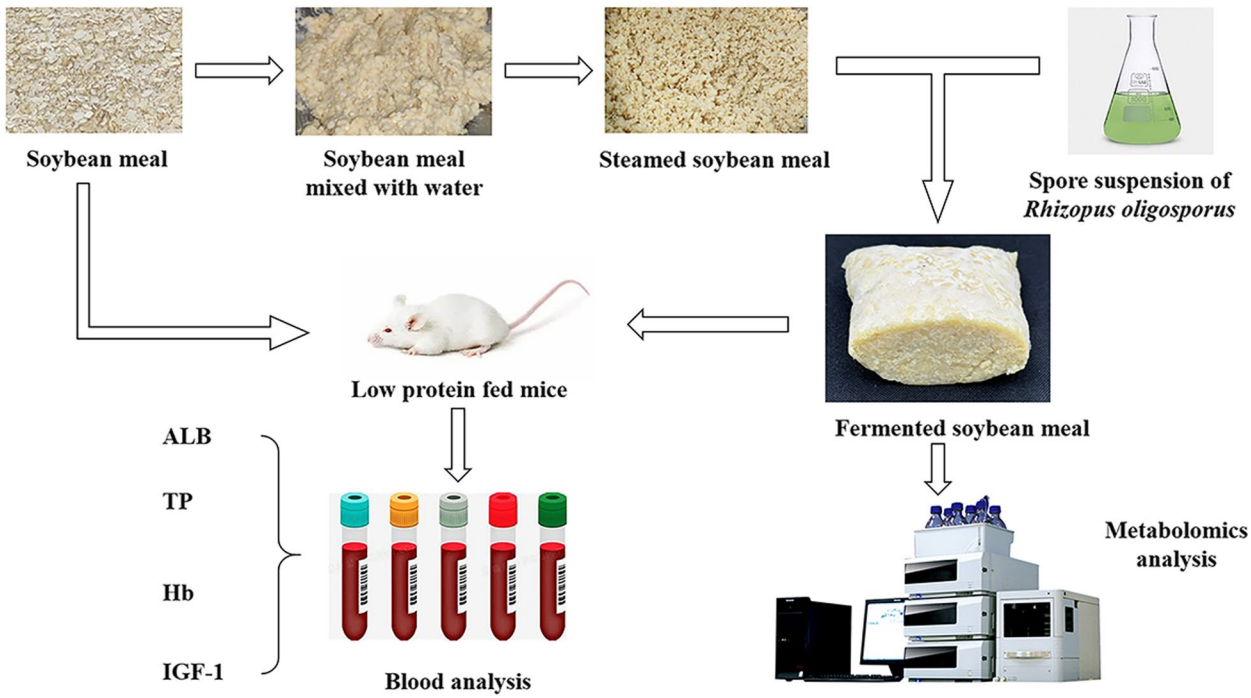
Graphical Abstract



Introduction

Soybean meal, also known as defatted soybean, is a byproduct of soybean oil production. It possesses a higher protein content compared to soybeans and is extensively used in high-protein products (Wang et al., 2021). Soybean meal can be categorized into high-temperature soybean meal and low-temperature soybean meal based on different processing temperatures. Low-temperature soybean meal refers to soybean meal that undergoes oil extraction at low temperatures or flash distillation, resulting in minimal protein denaturation and higher water solubility. It exhibits superior texture, color, and nutritional value compared to high-temperature soybean meal. Furthermore, it is a cost-effective and readily available agricultural and sideline product. However, in China, soybean meal is mostly used for livestock feed, such as raising poultry and pigs, with a small portion being utilized for cow feed and pet food production. Conversely, its application in food processing by factories remains limited, leading to inadequate and inefficient resource utilization of this high-yielding, nutritionally rich, and easily accessible raw-food material.

However, despite soybean meal being a high-protein food, its digestibility in vitro is only approximately 10% (Zhao et al., 2022). There are two primary reasons for this. Firstly, soybean protein is difficult to fully degrade into small peptides or amino acids and absorbed by the human body. Secondly, soybean meal contains anti-nutritional factors such as trypsin inhibitor, glycinin, phytic acid and oligosaccharide, further impairing its edible value (Kahala et al., 2023). To enhance the bioavailability of soybean meal, heat treatment and microbial fermentation (Chi et al., 2016) have been widely employed. These methods effectively reduce the content of anti-nutritional factors present in soybean meal or facilitate the degradation of soybean protein molecules into smaller peptides or amino acids. Zhang et al. (2017) demonstrated that the fermentation of soybean meal using a combination of *Lactobacillus*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* at 35 °C led to a reduction in trypsin inhibitor activity, an increase in free amino acid and small peptide content. In the initial stage of our study, steam heat treatment was applied to soybean meal, followed by inoculation with *Rhizopus oligosporus* to facilitate the fermentation process, resulting in a soybean meal fermentation product, which is similar to tempe. The noteworthy distinction between FSM and tempe lies in the almost non-existent fat content and the higher protein content of FSM, rendering it highly suitable for consumption among fitness enthusiasts.

Rhizopus oligosporus are usually used as fermentation strains in the production of fermented soybean products such as tempe. At present, the research on these products mainly focuses on nutrition and taste (Romulo et al., 2021; Rahmawati et al., 2021), but our understanding of the changes of their metabolites before and after the use of *Rhizopus oligosporus* for fermentation is still limited. Metabolomics can qualitatively and quantitatively analyze small and medium-sized molecular substances such as organic acids, fatty acids, amino acids, and sugars in samples (Booth et al., 2011). Targeted metabolomics is applicable to the study of known metabolic pathways or metabolites, while non-targeted metabolomics uses high-throughput analysis techniques to comprehensively and efficiently detect and identify all metabolites in the sample, in order to explore changes in a certain or a certain type of metabolite and its corresponding metabolic pathways. In the research related to food fermentation, non-targeted metabolomics has great potential. For example, some researchers (Ling et al., 2023) analyzed the metabolites and metabolic reactions during the fermentation of adzuki beans by *Pseudomonas acidophilus* using non targeted metabolomics and results showed that the fatty acid, biotin metabolism, and alkaloid biosynthesis pathways were significantly affected by fermentation, which helped promote the anti-obesity characteristics of adzuki beans. In a non-targeted metabolomics study, researchers discovered that okara produced 77 important bioactive compounds when fermented with *Neurospora crassa* strains NC-a and NC-b. These compounds contained mostly the flavonoids showed potential for targeting six major diseases (Yao et al., 2023). It can be seen that using non-targeted metabolomics methods can effectively solve the problem of analyzing metabolites in complex food systems during fermentation, discover changes in functional metabolites, and elucidate the biochemical mechanisms of related metabolic pathways. In our study, non-targeted metabolomics based on LC-MS was innovatively used in soybean meal fermented with *Rhizopus oligosporus* RT-3 and this tempe-like food could be an excellent resource of protein for human consumption.

Materials & methods

Materials and strain

Soybean meal was obtained from Yuwang Ecological Company, Shandong, China. The strain *Rhizopus oligosporus* RT-3 was preserved in the Food Microbiology Laboratory of Nanjing Agricultural University. Sodium hydroxide, acetaldehyde and trichloroacetic acid were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. LC-MS grade methanol (MeOH) was purchased from Fisher Scientific (Loughborough, UK), and 2-amino-3-(2-chlorophenyl) propionic acid was obtained from Aladdin (Shanghai, China).

Soybean meal fermentation process

A specific quantity of SM was weighed and mixed with double the amount of water while stirring. The mixture was stirred until uniform, followed by steam treatment for 20 min. After cooling to approximately 50 °C, 1% lactic acid and 1.5% *Rhizopus oligosporus* RT-3 spore suspension (concentration: 10^6 CFU/ml) were successively added, and the mixture was thoroughly mixed. The resulting mixture was then evenly laid flat into a square plastic bag, with a thickness of about 3 cm. Holes were pierced on both sides of the square, approximately 3 cm apart. The mixture was cultured in a 37 °C incubator for 24 h. After fermentation, the sample was immediately frozen at -80 °C for further analysis.

Nutritional changes during fermentation

Scanning electron microscope (SEM)

The freeze-dried samples of SM and FSM were adhered to aluminum sheets, and then gold-coated using an Eiko IB-5 ion sputter coater. The surface structure was observed using an EVO LS10 scanning electron microscope operated at an accelerating voltage of 10 kV.

Crude protein

Samples of SM and FSM were freeze-dried for further detection. Crude protein content was determined by Kjeldahl nitrogen analyzer (FOSS Kjeltec 8400) using a protein-to-nitrogen conversion factor of 6.25. d-Methionine (Sigma, CAS nr. 348-67-4) was used as a standard.

Peptide

Peptide water extract was obtained by the following methods: 5 g of freeze-dried fermented soybean meal powder and 50 mL of water were mixed and shaken for 1 h, followed by centrifugation at 10,000×g and 4 ℃ for 10 min. The supernatant was centrifuged again using the same conditions. Then 1 mL of the supernatant was mixed with 1 mL of 10% trichloroacetic acid solution, and allowed to stand for 30 min. The peptide extraction solution was obtained by centrifugation again under the same conditions. A 3 kDa ultrafiltration tube was used to centrifuge the peptide extraction solution at a speed of 10,000 × g for 10 min to obtain a peptide solution with a molecular weight less than 3 kDa.

Peptide content was determined by the biuret method which was subscribed as follows: The mixture of sample and water (1:10) was centrifuged at a speed of 8000×g for 10 min. After centrifugation, the supernatant was mixed with a 10% trichloroacetic acid solution and allowed to stand for 30 min. After centrifugation, 0.5 mL of the

supernatant was mixed with 2 mL of urea reagent and allowed to stand for 30 min. The absorbance value at 540 nm was measured. Construct a standard curve employing various concentrations of bovine serum protein as the reference substance. The standard curve equation is represented as $y = 0.0355x$ ($R^2 = 0.999$), where 'y' denotes the absorbance value and 'x' signifies the concentration of bovine serum protein (mg/mL).

Amino acid nitrogen

Sample weighted 0.3 g and 30 mL deionized water were mixed and shaken in a shaker for 1 h before centrifuged at 8000×g for 10 min. Then 70 milliliters of deionized water were added to 10 mL of supernatant followed by the gradual addition of 0.05 mol/L NaOH solution. Acetaldehyde solution (10 mL of 38%) was poured in when the pH reached 8.5. NaOH solution kept on dripping in until a pH of 9.2 was achieved and the volume of NaOH consumed was recorded and denoted as V. Perform a blank control titration on 80 milliliters of pure water, with the titration volume recorded as V_0. The final formula for calculating amino acid nitrogen was as follows:

$$X = (V - V_0) * c * 0.014)/(m * V_1/V_2) * 100$$

X - The content of amino acid nitrogen in the sample (g/100 g); c - Concentration of NaOH standard titration solution (mol/L); 0.014- The mass of nitrogen equivalent to 1.00 mL of NaOH standard titration solution [c (NaOH) = 1.00 mol/L], in grams (g); m - Weigh the mass of the sample (g); V_1- The amount of sample diluent taken (mL); V_2- Fixed volume of sample diluent, (mL); 100- Unit conversion factor.

Metabolomics analysis

Sample preparation for metabolomics analysis

An appropriate amount of SM or FSM was accurately weighed into a 2 mL centrifuge tube. Methanol of 600 µL containing 2-chloro-L phenylalanine (4 ppm) was added to the tube. The mixture was vortexed for 30 s, transferred to a tissue grinder, and steel balls were added. Grinding was performed for 60 s at 55 Hz, followed by ultrasonication for 15 min at room temperature. After centrifugation (10 min, 10000×g, 4 °C), the supernatant was collected and filtered through a 0.22 µm membrane. The filtrate was transferred to a detection bottle for LC-MS analysis.

LC-MS metabolomics analysis

An ultra-high-performance liquid chromatography system, ThermoVanquish (ThermoFisher Scientific, USA), was used with an ACQUITY UPLC® HSST3 (2.1×100 mm, 1.8 µm) chromatographic column (Waters, Milford, MA, USA). The flow rate was set at 0.3 mL/min with a column temperature of 40 °C, and the injection volume was 2 µL. In the positive ion mode, the mobile phase consisted of 0.1% formic acid acetonitrile (B2) and 0.1% formic acid water (A2), and a gradient elution procedure was employed: 0–1 min, 8% B2; 1–8 min, 8-98% B2; 8–10 min, 98% B2; 10–10.1 min, 98%-8% B2; 10.1–12 min, 8% B2. In the negative ion mode, the mobile phase consisted of acetonitrile (B3) and 5 mM Ammonium formate water (A3), and a gradient elution procedure was employed: 0–1 min, 8% B3; 1–8 min, 8-98% B3; 8–10 min, 98% B3; 10–10.1 min, 98%-8% B3; 10.1–12 min, 8% B3.

Mass spectrometry data were collected using a ThermoQExactive mass spectrometer (USA) equipped with an electric spray ion source (ESI). Data were collected in both positive and negative ion modes. The positive ion spray voltage was set at 3.50 kV, while the negative ion spray voltage was set at -2.50 kV. The sheath gas was set to 40 arb, and the auxiliary gas was set to 10 arb. The capillary temperature was maintained at 325 ℃. A first-order full scan was performed with a resolution of 70,000 over a mass range of m/z 100–1000. Second-order fragmentation was conducted using higher-energy collisional dissociation (HCD) at a collision energy of 30 eV. The second-order resolution was set to 17,500. The top 10 ions in the signal were selected for fragmentation, while dynamic exclusion was implemented to remove unnecessary MS/MS information.

Data analysis methods

The original mass spectrometry offline file was converted to mzXML file format using the MSConvert tool in the Proteowizard software package (v3.0.8789) (Smith et al., 2006). Peak detection, peak filtering, and peak alignment processing were performed using the RXCMS software package (Navarro-Reig et al., 2015) to obtain a quantitative list of substances and the result is shown in Supplementary Figs. 1 and 2. The following parameter settings were used: bw = 2, ppm = 15, peak width = c(5,30), mzwid = 0.015, mzdiff = 0.01, and method="centWave". Substance identification was carried out using public databases including HMDB (Wishart et al., 2007), Massbank (Horai et al., 2010), LipidMaps (Sud et al., 2007), mzcloud (Abdelrazig et al., 2020), KEGG (Ogata et al., 1999), as well as a self-built substance library. The parameters were set as ppm < 30 ppm. Total peak normalization, which normalizes the quantitative values of a single metabolite in the sample to the total quantitative values of all metabolites, was employed to achieve normalization, data correction, and elimination of system errors. A total of 7480 differential metabolites were identified from 15,650 metabolites, consisting of 3490 upregulated and 3990 downregulated differential metabolites.

Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed using the R software package Ropels (Thevenot et al., 2015) to perform dimension reduction analysis on the sample data (Supplementary Fig. 2). Permutation tests were conducted to assess the overfitting of the constructed models (Supplementary Figs. 4 and 5). R^2X and R^2Y represent the explanatory power of the models to the X and Y matrices, respectively. Q^2 indicates the predictive ability of the models, and higher values closer to 1 indicate better model fits and accurate classification of training set samples. Statistical tests were used to calculate *p*-values, and variable projection importance (VIP) values were determined using the OPLS-DA dimension reduction method. The fold change (FC) was calculated to assess the intensity and interpretability of each metabolome content on sample classification and discrimination, aiding in the screening of marker metabolites. Metabolite molecules were considered statistically significant when the *p*-value was <0.05 and the VIP value was >1. Based on the preset thresholds of *p*-value and VIP in statistical tests, it was found that out of the 339 secondary differential metabolites, 142 substances met the criteria of *p*-value <0.05 and VIP >1. These included 49 downregulated metabolites, 59 upregulated metabolites, and 34 metabolites with small fold changes ($-1<FC<1$). The remaining 231 metabolites did not show significant differences.

KEGG annotation and metabolic pathway analysis

The MetaboAnalyst software package (Xia & Wishart, 2011) was utilized to perform functional pathway enrichment and topology analysis of identified differential metabolic molecules. The enriched pathways were visualized using KEGG Mapper to facilitate browsing of differential metabolites and pathway maps.

The effects of SM and FSM on the growth of protein deficient mice

Animal diets

Animal feed was purchased from Jiangsu Collaborative Pharmaceutical Biotechnology Co., Ltd. The feed was prepared according to the recommendations of the American Institute of Nutrition for adult mice. Casein was used as the protein source. The control group feed contains 20% (wt/wt) of protein, while the low protein feed only contains 4% of protein. In addition, an equal amount of corn starch is used to replace the total amount of casein removed from the low protein diet formulation. The formula is shown in Table 1.

Table 1 The composition of animal diets

Ingredient	AIN-93G		4% Low protein feed	
	gm	kcal	gm	kcal
Casein	200	800	39.41	157.635
L-Cystine	3	12	0.59	2.3645
Corn Starch	397.5	1590	560.5	2242
Maltodextrin	132	528	132	528
Sucrose	100	400	100	400
Cellulose	50	0	50	0
Soybean	70	630	70	630
Vitamin Mix V10037	10	40	10	40
Mineral Mix S10022G	35	0	35	0
Choline Bitartrate	2.5	0	2.5	0
Total	1000	4000	1000	4000

Experimental animal conditions

Eighty male SPF grade BALB/c 4-week-old mice weighing 20 ± 2 g were purchased from the Experimental Animal Center of Nanjing Agricultural University with experimental license number NJAU No20230331038. The mice were subjected to a 7-day adaptive feeding period prior to the formal experiment. During the experiment, the environmental temperature was set at 25 ± 1 ℃, and the humidity was maintained at 40%±10%. The light/dark cycle was set at 12 h:12 h. The animal housing was pathogen-free, and the mice had free access to drinking water and feed throughout the experiment. The bedding was changed every week. All experiments were permitted by Ethics Committee of Nanjing Agricultural University and Laboratory Animal Welfare and the procedures were conducted in accordance with the "Regulations for the Administration of Laboratory Animals of the People's Republic of China" and the "Declaration of Helsinki of the World Medical Association".

Grouping and feeding of experimental animals

The experimental mice were divided into an experimental group and a blank control group, with a total of 11 groups. Random grouping was performed, with 5 mice per cage. Gavage was administered twice a day at 10 am and 4 pm. The specific groups were as follows:

(1) Blank control group: fed with regular feed throughout the experiment;
(2) Negative control group: fed with low-protein diet throughout the experiment;
(3) Positive control group: fed with low-protein diet for five weeks, followed by regular feed for five weeks;

(4) FSM 200 group: fed with low-protein diet and administered gavage with a dosage of 200 mg/kg.bw.d;
(5) FSM 100 group: fed with low-protein diet and administered gavage with a dosage of 100 mg/kg.bw.d.

During the experiment, the mice were observed daily for the condition of their eyes, mouth, and nose, as well as the state of their skin, fur, diet, water consumption, survival, activity, and stress response. During the bedding change, the secretion of urine and feces was observed to determine if there were any signs of toxicity in the mice.

Blood biochemical analysis

After the treatment cycle was completed, the mice were fasted overnight and their eyeballs were removed to obtain blood samples. The blood was collected into a clean, dry Tiger-Top tube without an anticoagulant to facilitate clotting at room temperature. After natural coagulation at room temperature for 1 h, the tube was centrifuged in a high-speed refrigerated centrifuge at 4 °C and 3500 r/min for 15 min to separate the serum and plasma. The samples were aliquoted according to the experimental requirements and stored at -20 ℃ for future use to avoid repeated cycles of freezing and thawing. The serum levels of ALB, TP, Hb, T-BIL, and IGF-1 were measured using a mouse Elisa kit following the steps outlined in the kit instructions.

Statistical analysis

All data were obtained from three independent experiments. Statistical significance was analyzed by ANOVA (SPSS version 25.0 for Windows). The differences between the means were determined by Duncan's test at an α level of 0.05.

Results and discussion

Nutritional improvement

After fermentation with *Rhizopus oligosporus* RT-3, the compact surface of the soybean meal became porous. As shown in Fig. 1, countless mycelium penetrated from the surface to the inside of soybean meal, which made full use of the substrate to grow. As the number of hyphae increased, *Rhizopus oligosporus* RT-3 secreted a large number of proteases that degraded the protein in soybean meal into small molecule peptides and amino acids. We could see from Fig. 1 that the content of crude protein (A), peptide (B) and amino acid nitrogen (C) showed a significant increase after fermentation. It was worth noticing that more than half of peptides had a molecular weight under 3 kDa, which had been reported to have higher biological activities (Liu et al., 2017).

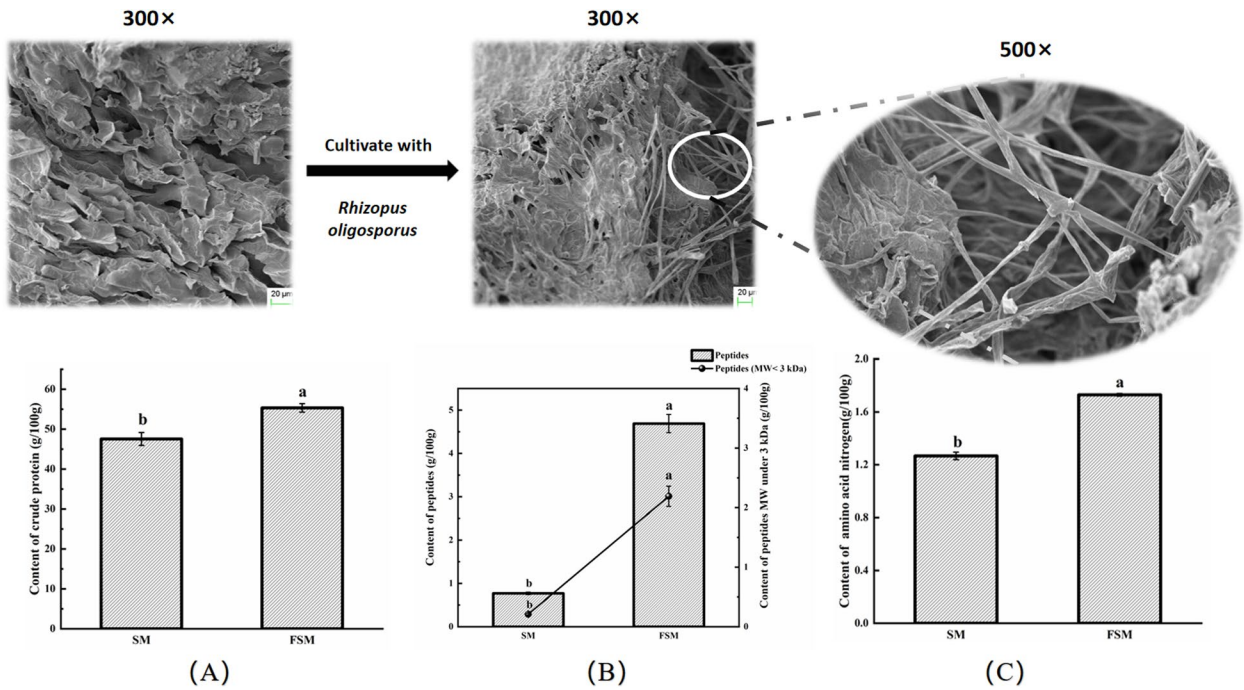


Fig. 1 Content changes after fermentation with *Rhizopus oligosporus* RT-3 and their SEM pictures. Legend: Figure **A**, **B** and **C** showed the content changes of crude protein, peptides and amino acids before and after fermentation respectively

Identification and analysis of differential metabolites in SM and FSM

A total of 142 differentially regulated secondary metabolites were classified, while 36 substances were not included in the classification. The remaining 106 substances were further grouped into 53 categories. Categories with at least three metabolites included amino acids, peptides, and analogues (20), carbohydrates and carbohydrate complexes (11), benzoic acid and its derivatives (4), fatty acids and conjugated fatty acids (4), linoleic acid and its derivatives (4), alcohols and polyols (3), amines (3), and Benzenediols (3). Significant changes appeared in amino acids, peptides, and carbohydrates before and after fermentation of soybean meal, suggesting that *Rhizopus oligosporus* likely utilized the sugars presented in soybean meal during its growth and produced proteases that broke down macromolecular proteins into small peptides and amino acids. On the other hand, the abundance of fatty acids was relatively lower, potentially due to the very low-fat content in the raw soybean meal itself. Therefore, despite the ability of *Rhizopus oligosporus* to generate lipase (Waseem et al., 2018), the resulting fatty acid profile of soybean meal after fermentation was relatively lower in quantity.

Table 2 displayed significant differential secondary metabolites between FSM and SM, as identified through the screening of VIP and *p*-values. The table provided detailed information on specific substances found in various metabolites, including their change trends and magnitudes. As depicted in Table 1, there were 20 amino acids, peptides, and analogues that experienced significant changes after fermentation, consisting of 12 up-regulated and 8 down-regulated substances. Notably, all up-regulated substances belonged to amino acids, including lysine, threonine, phenylalanine, which were essential amino acids for humans and could be obtained through protein macromolecule hydrolysis mediated by proteases. Conversely, the down-regulated products also fell within the category of amino acids exception for 4,5-dihydroorotic acid, possibly indicating their involvement in biosynthetic metabolic pathways. Another noteworthy group of metabolites was carbohydrates and carbohydrate conjugates, of which sucrose, mannitol, and D-glucose 1-phosphate displayed an upward trend, whereas the remaining substances exhibited a downward trend. These substances might serve as potential carbon sources required for the growth of *Rhizopus oligosporus*. Moreover, apart from these two mentioned metabolite classes, there were other up-regulated functional substances that warranted attention, such as pimelic acid associated with the citric acid cycle, α-linolenic acid, and aromatic compound methyl jasmonate.

Analysis of anti-nutritional factors

Based on the metabolomics data, we found that the content of stachyose and raffinose decreased to 18.1% and 8.5%, respectively, after fermentation. These two substances were always reported to have certain probiotic effects in the intestine (Mitamura et al., 2004; Li et al., 2013), but when it came to soybean meal and soybean meal fermentation, they were more often seen as anti-nutritional factors for causing symptoms of gastrointestinal bloating or abdominal pain such as intestinal ringing (Brasil et al., 2010). Since oligosaccharides are not considered heat-sensitive anti-nutritional factors, the heating treatment applied prior to fermentation can hardly have any effect on their content (Elango et al., 2022). Consequently, it could be inferred that fermentation significantly reduced the level of oligosaccharides in soybean meal, thereby enhancing its nutritional value. Currently, numerous studies focused on microbial fermentation to diminish oligosaccharide anti-nutritional factors, yet few explored the reasons for the reduction in content from a metabolic pathway perspective. Figure 2 illustrated the metabolic pathways associated with stachyose and raffinose during the process of *Rhizopus oligosporus* RT-3 fermentation of soybean meal. It was evident that stachyose and raffinose were converted into sucrose and melibiose under the influence of α-galactosidase and β-fructofuranosidase, resulting in a 3.67-fold increase and a 4.96-fold increase in their respective contents. Previous studies (K. et al., 1990) have shown that α-galactosidase aided in addressing gastric distention caused by stachyose and raffinose. Additionally, *Rhizopus oligosporus* could produce a substantial amount of α-galactosidase (up to 227 U/g) (Han et al., 2003). This further elucidated that one of the mechanisms by which *Rhizopus oligosporus* fermentation reduced oligosaccharide anti-nutritional factors was through the secretion of α-galactosidase, which promoted the conversion of stachyose and raffinose into sucrose, melibiose, and galactose.

Analysis of amino acids, peptides, and analogues

Soybean meal, as a byproduct of soybean oil pressing, is inherently rich in protein. Furthermore, *Rhizopus oligosporus* also produces a certain amount of fungal protein (Sar et al., 2022), further augmenting the protein content. During the fermentation process, the action of proteases significantly increased the levels of amino acids, polypeptides, and analogues in FSM, highlighting one of its key nutritional characteristics. KEGG enrichment analysis was conducted on the identified differential metabolites, revealing a total of 43 metabolic pathways with significant differences when considering a p-value < 0.5 and impact > 0.1. There were 22

Table 2 Differential metabolites between SM and FSM

Substance category	Substance name	m/z	Retention time(s)	log_2FC[a]	*P* value[b]	VIP[c]	Change
Alcohols and polyols	6β, 11α Dihydroxyprogesterone	347.22	369.7	3.65	6.29E-03	1.23	↑
	Inulobiose	325.11	404.8	1.96	5.77E-03	1.23	↑
	13,14-Dihydro-15-keto-PGE2	353.23	384.9	-1.83	2.17E-05	1.31	↓
Amines	Plant Sphingosine	318.3	450.8	3.61	6.64E-05	1.31	↑
	Spermidine	146.17	45.8	-1.64	1.18E-05	1.31	↓
	Oleoyl ethanolamide	326.31	513.8	-1.74	2.42E-02	1.15	↓
Amino acids, peptides, and analogues	L-Lysine	147.11	46.1	4.75	1.77E-02	1.27	↑
	N-acetyl Citrulline	217.11	285.1	4.65	3.45E-05	1.31	↑
	N-acetyl Histidine	198.08	55.3	3.59	1.97E-05	1.31	↑
	Saccharopine	277.14	55.2	3.07	6.05E-04	1.29	↑
	Histidine	154.06	52.3	2.69	3.73E-05	1.29	↑
	Phenylalanine	166.09	93	2.27	2.43E-03	1.26	↑
	Threonine	120.07	52.6	1.86	5.05E-05	1.31	↑
	N6 Acetyl L-lysine	187.11	74.3	1.85	1.71E-04	1.28	↑
	N-acetyl Glutaric acid	188.06	48.6	0.61	4.27E-02	1.07	↑
	Diaminopimelic acid	191.1	52.9	0.61	3.20E-02	1.12	↑
	γ- Glutamyl alanine	219.1	56.6	0.57	4.24E-03	1.24	↑
	Homoserine	100.04	45.6	0.29	2.17E-02	1.14	↑
	N-acetylornithine	175.11	54	-0.87	4.55E-03	1.3	↓
	Tyrosine	182.08	105	-0.56	1.68E-04	1.3	↓
	Proline	116.03	52.3	-6.29	4.49E-06	1.31	↓
	Glutamate	148.06	52.9	-1.35	2.46E-03	1.26	↓
	Aspartic acid	134.05	52.9	-3.4	7.99E-05	1.31	↓
	Arginine	173.1	64.4	-0.5	4.32E-03	1.23	↓
	Asparagus cochinchinensis	295.13	296.8	-3.03	5.94E-05	1.31	↓
	4,5-dihydro Orotic acid acid	158.96	228.1	-1.02	3.17E-02	1.12	↓
Benzoic acids and derivatives	Formylaminosalicylic acid	166.05	164.2	0.61	9.22E-03	1.21	↑
	Toluate	135.04	375.3	-3.07	2.43E-02	1.24	↓
	Phthalate	165.04	551.2	-4.82	8.40E-03	1.2	↓
	2-pyrocatechuic acid	153.02	245.1	-6.34	7.79E-03	1.28	↓
Carbohydrates and carbohydrate conjugates	Sucrose	343.3	412.2	3.67	1.03E-02	1.2	↑
	Mannitol	181.07	51.1	2.57	1.30E-03	1.26	↑
	D-Glucose 1-phosphate	259.02	45.6	2.2	8.29E-04	1.27	↑
	Trehalose	341.11	51.7	-1.44	1.17E-03	1.26	↓
	Sorbitol	182.98	454.3	-2.55	2.49E-03	1.31	↓
	Ribitol	151.06	52.3	-1.34	3.70E-04	1.28	↓
	Melezitose	504.17	355.3	-3.11	1.26E-07	1.32	↓
	Gluconic acid	195.05	48.4	-1.46	1.21E-05	1.3	↓
	Galactaric acid	209.03	44.9	-1.48	1.08E-03	1.27	↓
	D-Xylose	151.1	595.1	-2.32	2.35E-03	1.26	↓
	D-Arabitol	152.07	105.2	-3.34	2.73E-04	1.3	↓
Benzenediols	Norepinephrine	169.98	392	-1.78	3.31E-02	1.12	↓
	Hydroquinone	110.02	392	-1.15	1.28E-02	1.19	↓
	Epinephrine	184.1	116	0.23	7.84E-03	1.22	↑
Fatty acids and conjugated fatty acids	Heptanoic acid	113.06	211.5	5.65	1.39E-04	1.32	↑
	12,13-DHOME	315.25	472.4	1.06	1.63E-03	1.27	↑
	Palmitoleic acid	237.22	620.3	-3.02	9.11E-03	1.29	↓
	5-Hydroxypentanoic acid	118.07	445.7	-1.09	3.35E-03	1.25	↓

Table 2 (continued)

Substance category	Substance name	m/z	Retention time(s)	log_2FC^a	*P* value[b]	VIP[c]	Change
Lineolic acids and derivatives	Methyl jasmonate	207.14	541.5	4.3	1.49E-05	1.31	↑
	Alpha-Linolenic acid	261.22	682.5	3.96	3.93E-05	1.31	↑
	9-OxoODE	294.21	500.2	1.19	1.22E-02	1.2	↑
	(-)-Jasmonic acid	193.12	276.6	0.58	2.10E-03	1.26	↑

(↑) represents up-regulated after fermented, (↓) represents down-regulated after fermented

[a] FC means fold change

[b] VIP means variable importance in projection

[c] *P*-value means the significance of the difference between SM and FSM

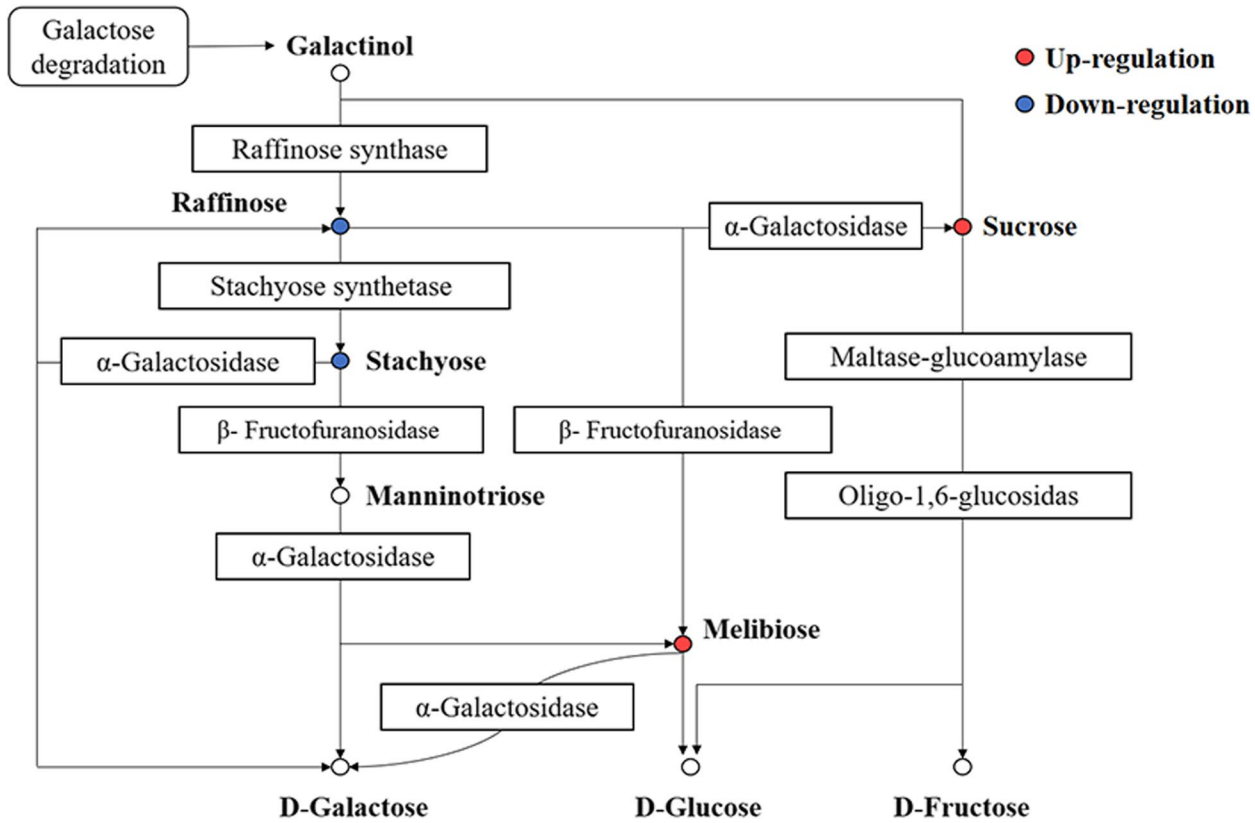


Fig. 2 Metabolic pathways related to stachyose and raffinose in soybean meal fermentation by *Rhizopus oligosporus* RT-3

metabolic pathways containing 5 or more metabolites and 10 out of them were associated with amino acids metabolism. Therefore, conducting further research on amino acids and peptides in FSM held considerable theoretical significance in guiding the development of functional products derived from it.

Analysis of amino acid metabolites

Cluster heatmap analysis The clustering heatmap of major amino acid metabolites in the differential metabolites of FSM and SM was presented in Fig. 3, offering a clear visualization of the upregulation and downregulation

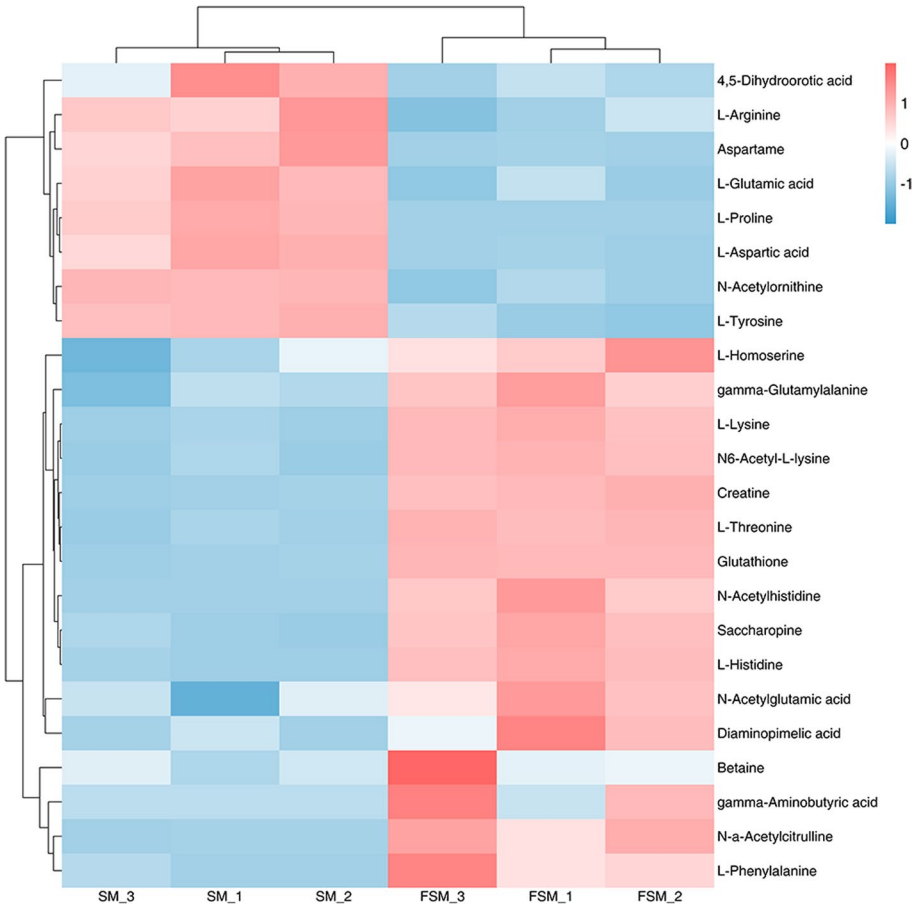


Fig. 3 Clustering heat map of main amino acids in FSM and SM differential metabolites

of each differential metabolite. From Fig. 3, it was evident that essential amino acids such as lysine, histidine, phenylalanine, and threonine experienced significant increases. Specifically, L-lysine exhibited an approximately 27-fold increase after fermentation, histidine increased 6.47-fold, phenylalanine increased 4.83-fold, and threonine increased 3.64-fold. These notable increases greatly enhanced the functionality and nutritional value of fermented soybean meal as a high-protein food source. Lysine in small quantities could promote the secretion of pepsin and gastric acid, improving stomach digestion and increasing appetite, thus supporting the growth and development of infants. Furthermore, lysine could be converted into sarcosine within the human body, and sarcosine, in turn, could be metabolized into creatine, which was stored in muscles and contributes to muscle growth, reduction of muscle fatigue, and improvement of athletic performance. Histidine, due to its unique chemical and metabolic properties, serves as a fundamental substance for the treatment of various diseases (Holecek, 2020). Phenylalanine acted as a precursor to dopamine and norepinephrine, essential neurotransmitters that could be synthesized through biochemical transformations, improving human excitability, enhancing exercise capacity and learning ability, and participating in glycolipid metabolism activities essential for human energy supply (Hong et al., 2021). Moreover, non-protein amino acids such as betaine, creatine, and γ-aminobutyric acid were also significantly upregulated with upregulation factors of 3.5, 5.9, and 6.0, respectively. This suggested that the antioxidant, energy metabolism regulation, and sleep quality improvement functions of FSM (Luo et al., 2021; Kheradmand et al., 2013; See et al., 2012) were enhanced to a certain extent.

KEGG enrichment analysis Comparing the 142 differential metabolites of FSM and SM with the KEGG database revealed that out of the 187 metabolic pathways containing these differential metabolites, 64 metabolic pathways showed significant differences when considering a p-value < 0.05. Among these, 14 metabolic pathways were found to be related to amino acids. The specific metabolic pathways were presented in Fig. 4. The differences in amino acids before and after fermentation mainly revolved around the TCA cycle. Figure 4 demonstrated that after fermentation, there was a decrease in citrate content and

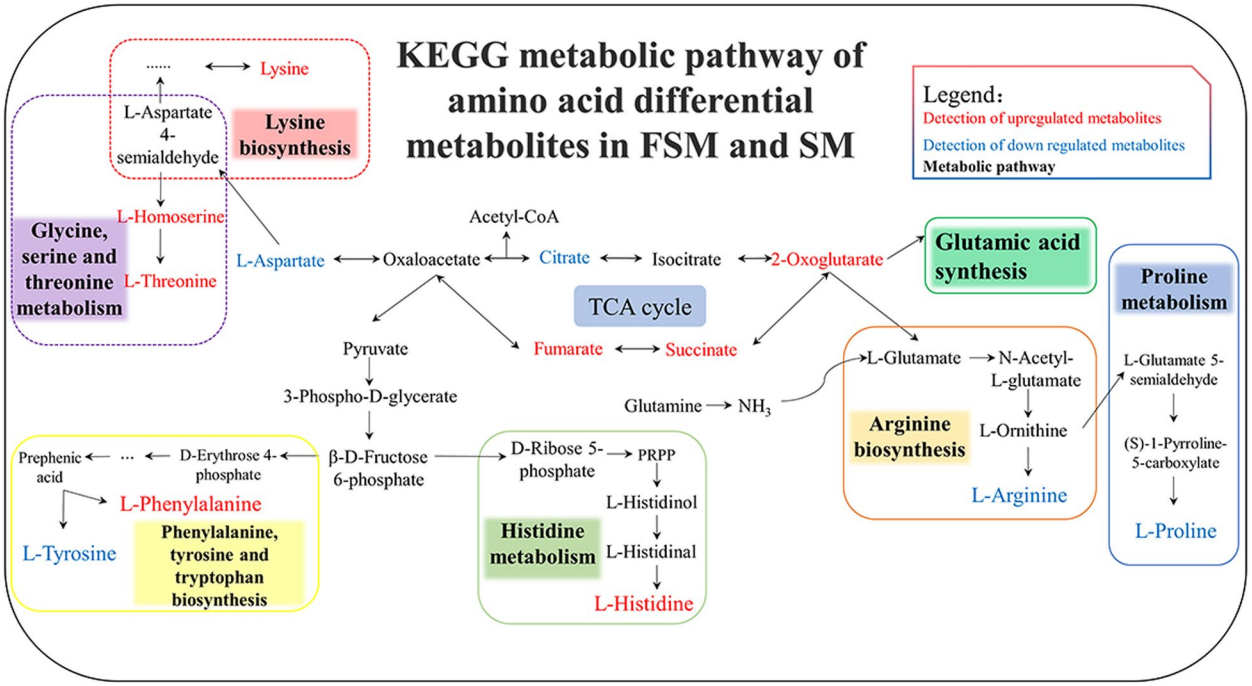


Fig. 4 KEGG pathway analysis of differential metabolites about amino acids before and after soybean meal fermentation

an increase in 2-oxoglutarate, succinic acid, and fumaric acid. 2-oxoglutarate was involved in the synthesis of glutamic acid and arginine, and ornithine indirectly participates in proline metabolism in the synthesis of arginine. Citrate was converted to oxaloacetic acid through the TCA cycle, and oxaloacetic acid could further convert to threonine and aspartic acid, which were key substances for lysine synthesis, indirectly promoting the upregulation of lysine and threonine. Pyruvic acid obtained from the conversion of oxaloacetic acid was converted to fructose 6-phosphate through a series of reactions, which was involved in the biosynthesis of phenylalanine and tyrosine. Additionally, fructose 6-phosphate was involved in histidine metabolism through its conversion to ribose 5-phosphate, leading to increased histidine content after fermentation. Compared to soybean meal before fermentation, the KEGG metabolic pathway analysis also demonstrated significant differences in metabolites of FSM, indicating the participation of *Rhizopus oligosporus* RT-3 in the metabolic pathway of soybean meal and its impact on the increased or decreased content of metabolites.

Analysis of peptide metabolites

Before and after fermentation, a total of nine peptide metabolites were identified, of which all but glutathione belonged to the category of dipeptides. The results obtained from comparing these nine peptide sequences with the BIOPEP database of bioactive peptides were presented in Table 3. It was evident that the biological functions of the polypeptide metabolites primarily revolved around blood pressure reduction and blood sugar regulation, which aligned with the biological functions of peptides found in tempe. Some researchers (Tamam et al., 2019) employed metabonomics to investigate the variations in tempe produced by three

Table 3 Differential metabolites of peptides in FSM, SM and their biological activities

Name	Exact mass	Bioactivities[a]
Alanyl-Proline	186.10	DPP IV inhibitor
		ACE inhibitor
Aspartyl-Glutamate	262.08	DPP IV inhibitor
		ACE inhibitor
Cysteinyl-Glycine	178.04	uk
D-Alanyl-D-alanine	160.08	Hypotensive peptide
		DPP IV inhibitor
		ACE inhibitor
Glutathione	307.08	Antioxidative
		ACE inhibitor
Glycyl-glycine	132.05	DPP IV inhibitor
		ACE inhibitor
Glycyl-leucine	188.12	DPP IV inhibitor
		ACE inhibitor
Histidinyl-Serine	242.10	DPP IV inhibitor
Tyrosyl-Alanine	252.11	DPP IV inhibitor

[a] ACE inhibitor is for antihypertensive; DPP IV inhibitor is for antidiabetic; uk means unknown

factories with differing levels of cleanliness in Indonesia. Their study revealed that among the identified peptide segments in tempe samples from the three factories, 26, 21, and 26 peptide segments were chiefly associated with ACE inhibitors (as antihypertensive agents) and DPP-IV inhibitors (as antidiabetic agents). These functional peptides were predominantly dipeptides, and majority of the peptides with a length greater than tripeptide lack any annotation regarding their biological activity or function in BIOPEP. Other researchers (Sitanggang et al., 2020) used water, alkaline protease, and papain to extract polypeptides from tempe after 144 h of fermentation and compared them with the BIOPEP database, resulting in the identification of 10, 12, and 12 types of polypeptides with main biological functions as ACE inhibitors, antioxidants, and antithrombotics, respectively. This suggested that although soybean meal differs from soybean in origin and variety, the polypeptides generated through protein degradation exhibit certain similarities in biological function after fermentation by *Rhizopus oligosporus*. Additionally, the polypeptide chains produced by FSM were shorter and more extensively degraded.

Improvement of protein malnutrition mice growth by soybean meal and fermented soybean meal

As we expected, the total protein and albumin content in the control group were lower than those in the mice receiving a normal diet as shown in Fig. 5A and B. Total protein represents the sum of all proteins in the plasma, including albumin and globulin. Changes in total protein levels can reflect the overall protein metabolism status in the body, while albumin plays a crucial role in maintaining plasma osmotic pressure and transporting nutrients to the tissues (Wei et al., 2023). After feeding mice with high and low doses of FSM, the levels of total protein and albumin in the blood could be restored to the levels of the normal diet group, significantly higher than those in mice on a low-protein diet. Similarly, HB and IGF-1 decreased after long-term low-protein diets (Fig. 5C and D). Following FSM feeding, HB in the blood was significantly higher than that in the normal diet group, indicating the potential presence of key factors in FSM regulating hemoglobin content growth, thus enhancing the organism's capacity for oxygen transport and reducing the risk

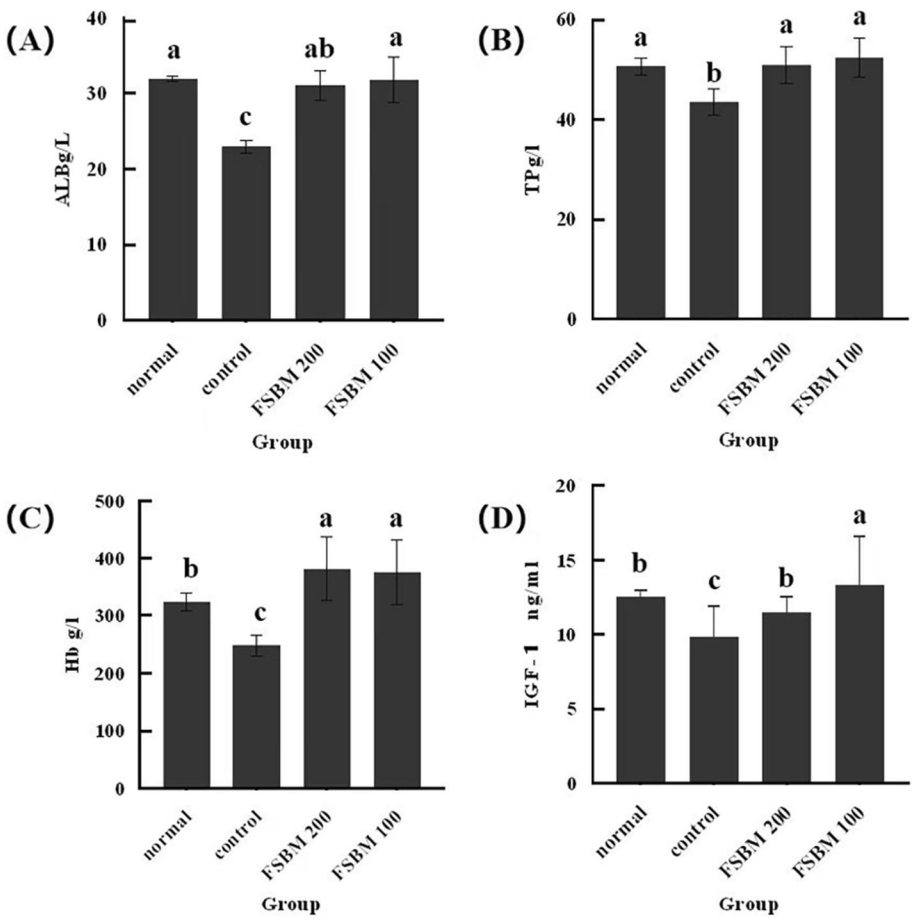


Fig. 5 Growth status indicators of mice fed with different doses of FSM. Legend: Figure **A**, **B**, **C** and **D** showed the levels of ALB, TP, HB, and IGF-1 in the blood of mice respectively

of anemia. IGF-1 is typically associated with growth and developmental status. It was noteworthy that the low-dose FSM feeding group exhibited a significant increase compared to the high-dose feeding group, suggesting the possible presence of a phenomenon of nutrient excess, requiring attention to intake control.

Conclusions

In summary, the metabonomic analysis of soybean meal before and after fermentation demonstrated the significant impact of *Rhizopus oligosporus* RT-3 on the metabolic process of soybean meal. Compared to unfermented soybean meal, the fermentation process with *Rhizopus oligosporus* RT-3 led to a significant increase in crude protein, peptides, and amino acids and decrease in the content of oligosaccharide anti-nutritional factors, such as raffinose and stachyose which were hydrolyzed by α-galactosidase and β-furofuranosidase. It was observed that amino acid metabolites, particularly essential amino acids such as lysine, histidine, and threonine, were up-regulated. The polypeptide metabolites were basically identified as dipeptides, with their biological activities mainly concentrated in ACE inhibitors and DPP-IV inhibitors. Factors such as IGF-1, HB, ALB and TP showed significant increases in mice feeding FSM compared to low- protein feeding mice, which represented the ability of FSM to improve growth condition. This study highlighted that fermentation with *Rhizopus oligosporus* RT-3 not only reduced the content of anti-nutritional factors but also degraded macromolecular proteins into small amino acids and various functional peptides, thereby enhancing the digestibility and nutritional value of soybean meal. Moreover, by converting soybean meal, a byproduct of soybean processing, into a high-protein and low-fat functional food ingredient, FSM not only held significant economic value but also aligned with the principles of food resource conservation and ecological environment protection, which possessed high significance and potential for further development.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43014-024-00286-w.

Supplementary Material 1: Supplementary Figure 1. Base peak chromatogram of SM and FSM in positive mode

Supplementary Material 2: Supplementary Figure 2. Base peak chromatogram of SM and FSM in negative mode

Supplementary Material 3: Supplementary Figure 3. PCA (fig.A, fig.B), PLS-DA (fig.C, fig.D) and OPLS-DA (fig.E, fig.F) of FSM and SM. Legend: Fig.A, fig.C and fig.E are in positive mode while fig.B, fig.D and fig.F are in negative mode

Supplementary Material 4: Supplementary Figure 4. Permutation tests of PLS-DA (fig.A) and OPLS-DA (fig.B) in positive mode

Supplementary Material 5: Supplementary Figure 5. Permutation tests of PLS-DA (fig.A) and OPLS-DA (fig.B) in negative mode

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Not applicable.

Authors' contributions
Teng Sun: Conceptualization, Formal analysis, Methodology, Validation, Writing - Original Draft, Visualization; Zhaojun Wang: Methodology, Validation, Visualization; Yiqiang Dai: Writing - Review & Editing, Visualization; Kang Zhai: Validation, Visualization; Xiangna Fang: Validation, Visualization; Yitian Chen: Visualization; Baomin Zhao: Resources; Mingsheng Dong: Conceptualization, Methodology, Writing - Review & Editing, Visualization, Supervision.

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Data availability
The datasets during and/or analysed during the current study available from the corresponding author on reasonable request and part of the data generated or analysed during this study have been included in the supplementary information files.

Declarations

Ethics approval and consent to participate
This material is the authors' own original work, which has not been previously published elsewhere.
The paper reflects the authors' own research and analysis in a truthful and complete manner.
The study was reviewed and approved by Ethics Committee of Nanjing Agricultural University and Laboratory Animal Welfare. All experiments followed the guide for the "Regulations for the Administration of Laboratory Animals of the People's Republic of China" and the "Declaration of Helsinki of the World Medical Association".

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]College of Food Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China. [2]Jiangsu Palarich Food Co., Xuzhou, Jiangsu, China.

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