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Effect of Different Diets on the Hepatopancreatic Proteomes of Chinese Mitten Crab (*Eriocheir Sinensis*)

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Abstract

Aquatic plants and freshwater snails are important natural food sources of *Eriocheir sinensis*. The effects of these two kinds of natural food sources on the growth and development of *Eriocheir sinensis* were studied by determining the hepatopancreatic proteomes of three crab groups, namely, crabs fed with aquatic plants combined with freshwater snails (group A), crabs fed with aquatic plants only (group B), and crabs fed with freshwater snails only (group C), with tandem mass tag technology. Results showed 110 differentially expressed proteins between groups A and B, among which 78 were up-regulated and 32 were down-regulated in group A. Meanwhile, 9 proteins were up-regulated and 14 proteins were down-regulated in group A relative to those in group C. The proteins related to molting and growth that were differentially expressed between groups A and B were up-regulated in group A. These proteins included cryptocyanin and cuticle protein CBM. The immunity-related proteins, such as mannosyl-oligosaccharide glucosidase and glutathione peroxidase, that were differentially expressed between groups A and C and were up-regulated in group A. These results indicated that freshwater snails might promote the growth and development of *E. sinensis* to a certain extent, and aquatic plants might play an important role in the immunity of *E. sinensis.* Our study provides a theoretical basis for the practice of “planting grass and throwing snails” in the green ecological culture of *E. sinensis.*

Introduction

Chinese mitten crab (*Eriocheir sinensis*), commonly known as river crab, is an important economic aquaculture species in China (1). The planting of aquatic plants has become one of the key factors for the success of crab culture. Aquatic plants can not only regulate the pH of water and provide shelter to crabs, they can also be used as food sources by crabs due to their rich nutrient contents (2, 3). Several researchers have indicated that aquatic plants are beneficial for crab growth and can improve the nutritional quality of the edible parts of crabs. Crabs ingest a certain amount of aquatic plants to meet their nutritional needs even when receiving sufficient feed (4). Freshwater snails are a high-quality natural feed for crabs, and snail feeding in the process of crab culture can increase breeding yield and improve quality (5, 6). Snails, as an animal feed, can increase the content of animal protein; this effect has a certain influence on crab growth (7).

The growth and development of organisms are closely related to food sources. Different food sources cause changes in the composition of proteins in tissues and organs; affect biological processes, such as digestion and absorption, energy metabolism, and immune response; and further affect the growth and development of organisms (8, 9). Comparing the liver proteomes of rats fed with animal protein with those of rats fed with plant protein revealed that the two groups exhibited drastic changes in their protein expression profiles and considerably different amino acid metabolism and fatty acid metabolism (10). The content of proteins related to lipid, carbohydrate, and amino acid metabolism changed in the livers of *Oreochromis niloticus* fed with diets containing different nutrients, and the immune systems of the test organisms also changed (11). Replacing dietary fish oil with linseed oil, resulted in considerable changes in the protein expression profile in the hepatopancreas of *E. sinensis*; subsequently, the capability of this

crab species to adapt to the environment was also altered (12). Food sources can obviously affect the protein composition of organisms and then further affect the growth and development of organisms. Aquatic plants and freshwater snails play an important role in the growth and development of crabs as two kinds of important natural food. However, research on how aquatic plants and snails affect the growth and development of crabs remains scant.

A comprehensive analysis of the composition and dynamics of proteins offers important insights into the roles of aquatic plants and snails in the process of crab culture. Therefore, in this study, three diverse feed types were provided as daily crab diets: freshwater snail (*Sinotaia quadrata*); waterweed plants (*Elodea canadensis*); and a mixed diet of *S. quadrata* and *E. canadensis*. Then, the protein profiles of crabs under the three different feed types were determined and compared to investigate the effect of aquatic plants and snails on crab growth and development.

Materials And Methods

Sample collection and ethics statement

Juvenile crabs (approximately 7.5 g) with similar growth conditions were collected from the aquatic animal germplasm resource station of Shanghai Ocean University. They were fed in a circulating water system for 7 days to adapt to the environment. Then, the crabs were randomly divided into three groups and fed as follows: Group A was fed with a mixed diet of *S. quadrita* and *E. canadensis*, group B was fed with *E. canadensis* only, and group C was fed with *S. quadrita* only. All the crabs were reared in the “Crab Dragon Palace” in the same environment. The water temperature was maintained at 26 ℃ ± 2 ℃, and the three groups were fed with the same amount of food at 9:00 every day. When the crabs grew to the early stage of molting, their hepatopancreas tissues were collected. Three biological repeats were set for each group. Then, the tissues were quickly frozen in liquid nitrogen and stored in a −80 ℃ refrigerator. The whole process follows the institutional animal care and use committee of Shanghai Ocean University (Shanghai, China).

Protein extraction and quality control

The collected hepatopancreatic tissues were removed from −80 °C refrigerator and homogenized. Approximately 50 mg of minced tissue was mixed with 500 μl of RIPA lysate (PMSF was added before use). Subsequently, the homogenate was incubated in an ice bath for 30 min. Centrifugation was performed at 14 000 g for 10 min at 4 °C. The supernatant was collected in a new tube. Protein concentration was measured with a Pierce BCA protein assay kit in accordance with instructions (Thermofisher, USA), and protein quality was tested through SDS-PAGE gel electrophoresis.

Protein alkylation, trypsin enzymatic hydrolysis, and TMT tagging

The proteins were alkylated in accordance with Randall (13), and the filter-aided proteome preparation method was used for protease hydrolysis (14). The trypsin enzyme was added on the basis of the ratio of

protein: enzyme = 40:1. The mixture was placed at 37 °C overnight. Then, the peptide segment was desalted and lyophilized. A total of 100 μg of protein was taken from each sample for TMT labeling. The labeling steps are as follows: First, the temperature of the TMT reagent was allowed to recover to room temperature. Then, acetonitrile was added to the sample, and the sample was centrifuged at low speed with a vortex. Second, the sample was mixed with TMT reagent, incubated at room temperature for 2 h, and then mixed with hydroxylamine. The mixture was reacted at room temperature for 15 min. Finally, the same amount of labeled substances was mixed in a tube and drained with a vacuum concentrator.

HPLC fractionation and LC–MS/MS analysis

Polypeptide samples were redissolved with UPLC loading buffer, and a reverse phase C18 column was used to separate the high pH liquid phase. A total of 20 fractions were collected and merged into 10 fractions in accordance with peak type and time. After vacuum centrifugation and concentration, the mass spectrometry sample was dissolved with the loading buffer solution for mass spectrometry. The mass spectrometry conditions were as follows: The data acquisition software was Thermo Xcalipur 4.0 (Thermo, USA). The chromatographic instrument was Easy NLC 1200 (Thermo, USA), and the mass spectrometer was Q_Exactive HF-X (Thermo, USA). The chromatographic separation time was 120 min, the flow rate was 300 nl/min, the scanning range of MS was 350–1300 m/z, and the acquisition mode was DDA.

Bioinformatic analysis

ProteomeDiscovererTM software 2.4 was used to search NCBI and the Uniprot database to identify and quantify proteins. Proteins with fold change (FC) < 0.667, FC >1.5, and $P < 0.05$ were considered as differentially expressed proteins. Differentially expressed proteins were subjected to GO and KEGG enrichment analysis by using the software implemented in Majorbio I-Sanger Cloud Platform with corrected $P < 0.05$.

Results

Overview of total identified proteins

After submitting the original data file of the mass spectrometer off machine to the Proteome Discoverer server, 358,336 secondary spectra were obtained, 67,127 spectra were matched, and 24,744 peptide fragments and 9,959 proteins were identified (Fig. 1). After the functional annotation of the identified proteins, 4,277 annotated proteins were obtained. Among these proteins, 2,532 proteins were annotated in GO enrichment, accounting for 59.2% of the total annotated proteins, and 3,041 were annotated KEGG pathway, accounting for 71.1% of the total annotated proteins (Table 1). These results indicated that the proteomic data of this study were reliable.

Table 1
The information of protein GO and KEGG annotation.

Databases	Number of proteins	Percentage
SubCell-Location	4277	1
COG	1481	0.3463
KEGG	3041	0.711
GO	2532	0.592
Pfam	3844	0.8988
Total	4277	1

Bioinformatic analysis of differentially expressed proteins

The hepatopancreatic proteomics of the three groups were compared and analyzed. A total of 323 differentially expressed proteins were identified with the statistical thresholds of $P < 0.05$, FC > 1.5, or FC < 0.67. Compared with those in group B, 78 proteins were highly expressed and 32 proteins were expressed at low levels in group A. Compared with those in group C, 9 were highly expressed and 14were expressed at low levels in group A (Fig. 2).

GO enrichment analysis indicated that the differentially expressed proteins between groups A and B were mainly enriched in hydrolase activity, deacetylation, lipoprotein metabolism, and galactosidase activity. The differentially expressed proteins between groups A and C were mainly enriched in oxidative stress reaction and amino acid metabolism (Fig. 3). KEGG database was used to analyze the enrichment of differentially expressed proteins in metabolic pathways. The results showed that the differentially expressed proteins between groups A and B were mainly enriched in lysosomes, sphingolipid metabolism, and polysaccharide degradation pathways, whereas the differentially expressed proteins between groups A and C were mainly enriched in metabolic pathways related to infection (Fig. 4).

Differentially expression proteins between groups

The proteomes of groups A and B were compared and analyzed. The proteins that were highly expressed in group A included cryptocyanin, cuticle protein, solute carrier family 35 member F6, programmed cell death protein, fibroblast growth factor receptor 3. The proteins expressed at low levels in group A were ataxin-2, metalloreductase, pancreatic lipase-related protein 2, and arylsulfatase A (Table 2). The proteomes of groups A and C were also compared and analyzed. Mannosyl oligosaccharide glucosidase, glutathione peroxidase 2, calreticulin were among the proteins that were highly expressed in group A relative to group C. The proteins that were expressed at low levels in group A were myosin and Rho-associated protein kinase 2 (Table 3).

Table 2
Important differentially expressed proteins between the hepatopancreas of *E. sinensis* in group A fed with *E. canadensis* and *S. quadrata* and in group B fed with *E. canadensis* only ($P < 0.05$, FC < 0.67, or FC > 1.5,).

Protein ID	**Protein name**	**Annotation**	**Fold change A/B**	**Related Function**
A0A223G1B9	Cryptocyanin	Putative hemocyanin	3.83	Molt and growth
O96992	Cryptocyanin	Putative hemocyanin	6.67	Molt and growth
A1YLE8	Cuticle protein CBM	Cuticle protein CBM	6.69	Cuticle
Q8N357	SLC35F6	Solute carrier family 35 member F6	1.70	Cell proliferation
P47816	PDCD2	Programmed cell death protein 2	1.77	Cell proliferation
Q99KD5	UNC45 homolog A	Protein unc-45 homolog A	1.73	Cell proliferation
O00571	DDX3X	ATP-dependent RNA helicase DDX3X	1.51	Cell growth
Q14678	KANK1	KN motif and ankyrin repeat domain-containing protein 1	1.50	Cell proliferation
Q9I8 × 3	FGFR3	Fibroblast growth factor receptor 3	1.90	Cell proliferation
Q6NX65	PDCD10	Programmed cell death protein 10	1.76	Cell proliferation
O70305	Atxn2	Ataxin-2	0.46	Negative regulation of growth
Q687 × 5	STEAP4	Metalloreductase	0.64	Negative regulation of cell proliferation
P12617	MLYCD	Malonyl-CoA decarboxylase	1.60	Lipid synthesis
Q99JB2	Stoml2	Stomatin-like protein 2	1.62	Lipid localization
A0A0P4VPE9	None	FABP domain-containing protein	1.58	Lipid binding
Q9NUQ2	AGPAT5	1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon	1.50	Phospholipid synthesis
P57093	Phyh	Phytanoyl-CoA dioxygenase	1.65	Lipid metabolism
Q91XV4	DCXR	L-xylulose reductase	0.60	Carbohydrate metabolism

Protein ID	Protein name	Annotation	Fold change A/B	Related Function
Q95327	MANBA	β-mannosidase	0.47	Carbohydrate metabolism
P54318	Pnliprp2	Pancreatic lipase-related protein 2	0.32	Nutrition response
P50428	Arsa	Arylsulfatase A	0.58	Nutrition response

Table 3
Important differentially expressed proteins between the hepatopancreas of *E. sinensis* in group A fed with *E. canadensis* and *S. quadrata* and group C fed with *S. quadrata* only ($P < 0.05$, FC < 0.67, or FC > 1.5,).

Protein ID	Protein name	Annotation	Fold change A/B	Related Function
Q80UM7	MOGS	Mannosyl-oligosaccharide glucosidase	1.52	Immune
Q4AEI0	GPX2	Glutathione peroxidase 2	1.62	Immune
A0A193DUV8	Calreticulin	Calreticulin	4.50	Immune
P05661	Myosin	Myosin heavy chain	0.61	Muscle contraction
M3TYT0	R0CK2	Rho-associated protein kinase 2	0.64	Muscle contraction

Discussion

Aquatic plants and freshwater snails are important natural food sources for crabs and have a direct effect on crab growth and development (15, 16). This study was conducted to investigate the differences in the hepatopancreatic proteomes of crabs under three different feeding methods: aquatic plants combined with freshwater snails (group A), aquatic plants only (group B), and freshwater snails only (group C). The results showed differences in the protein expression profiles in the hepatopancreas of crabs among the three groups. A total of 110 proteins were differentially expressed between groups A and B, whereas only 23 proteins were differentially expressed between groups A and C. Freshwater snails might affect the protein expression profiles of crabs more than aquatic plants.

Among the differentially expressed proteins between groups A and B, the proteins with high expression levels and the most significant differences in group A were cryptocyanin and cuticle protein CBM. Cryptocyanin is an important member of the hemocyanin gene family and a crustacean molting protein

that is closely related to molting and plays an important role in new exoskeleton formation after molting (17, 18). Cuticle protein is an important component of the crab exoskeleton. During molting, the old epidermis falls off, and a new epidermis form. Cuticle protein plays a vital role in the formation of the epidermis during molting (19). Cryptocyanin and cuticle protein expression levels were significantly higher in crabs fed with aquatic plants and freshwater snails than in those fed with aquatic plants only, indicating that the molting frequency of crabs fed with aquatic plants and freshwater snails might be accelerated; this result was consistent with our previous research results showing that the molting rate of crabs fed with aquatic plants and freshwater snails is significantly faster than that of crabs fed with aquatic plants only (20). The results of this study showed that the addition of snails to crab diets could affect the expression of molting-related proteins and further affect the molting rate. A large number of proteins related to cell proliferation and growth were highly expressed in crabs fed with aquatic plants and freshwater snails; these proteins included solute carrier family 35 member F6, programmed cell death protein 2, UCN-45 protein homolog A, and fibroblast growth factor receptor 3 (21, 22) (Table 2). The high expression of these proteins leads to the increase in cell number and volume and further affects crab growth and development. Therefore, crabs fed with aquatic plants and freshwater snails grew significantly faster than crabs fed with aquatic plants.

Among the proteins that were differentially expressed between groups A and C, those that were highly expressed in group A were mannosyl oligosaccharide glucosidase, glutathione peroxidase-2, and calreticulin. Mannosyl oligosaccharide glucosidase is involved in the metabolism of mannan oligosaccharides and can improve immunity in *Litopenaeus vannamei* (23, 24). Glutathione peroxidase plays an important role in immune defense against pathogen infection in invertebrates. Research on *Haliotis discus*, *Chlamys farreri*, *L. vannamei*, and *Fenneropenaeus chinensis* has shown that glutathione peroxidase is involved in the immune regulation process (25–28). Calreticulin is a highly conserved calcium binding protein, which is an immune-related protein in vertebrates and invertebrates. Studies on *Patinopecten yessoensis*, *Sebastes schlegeli*, and *Tilapia niloticus* all showed that calreticulin is involved in immune function (29–31). The levels of these proteins were higher in crabs fed with aquatic plants and freshwater snails than in crabs fed with only freshwater snails, suggesting that aquatic plants might affect crab immunity; moreover, these results were consistent with previous results that submerged plants in the diet can enhance crab immunity (32, 33).

In conclusion, diets containing aquatic plants can enhance crab immunity, while those containing freshwater snails can promote crab growth and molting. A mixed diet containing aquatic plants and freshwater snails is the best choice for crabs. The results of this work provide a theoretical basis for the practice of “planting grass and throwing snails” in green crab ecological culture.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data used to support the findings of this study are available upon request to the corresponding author. The dataset generated in this study has been deposited in the ProteomeXchange Cosortium (http://proteomecentral.proteomeexchange.org) with the dataset identifier px-submisssion #468676.

Competing interests

All authors declare that they have no competing interests.

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Authors' contributions

JW and CHW conceived the original idea of the study; XWC, XH, ZHL and DYY performed experiments; JW, and XWC analyzed data; XWC and XH interpreted results; XWC and XH prepared figured and drafted manuscript; JW and CHW edit and revised manuscript. XWC and XH approved final version ofmanuscript. XWC and XH contributed equally to this work.

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Figures

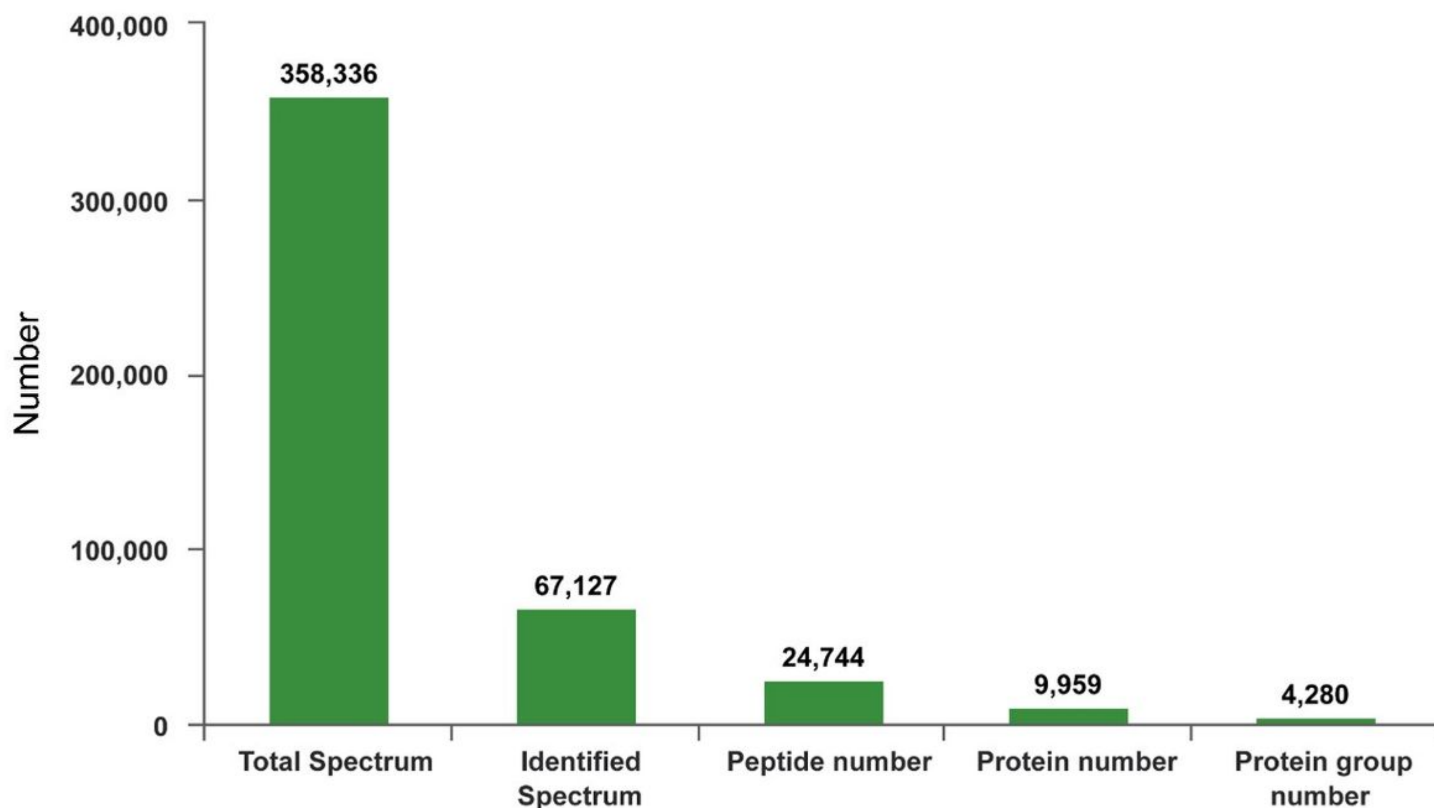


Figure 1

Statistical data of reliable peptides in hepatopancreas samples of E. sinensis.

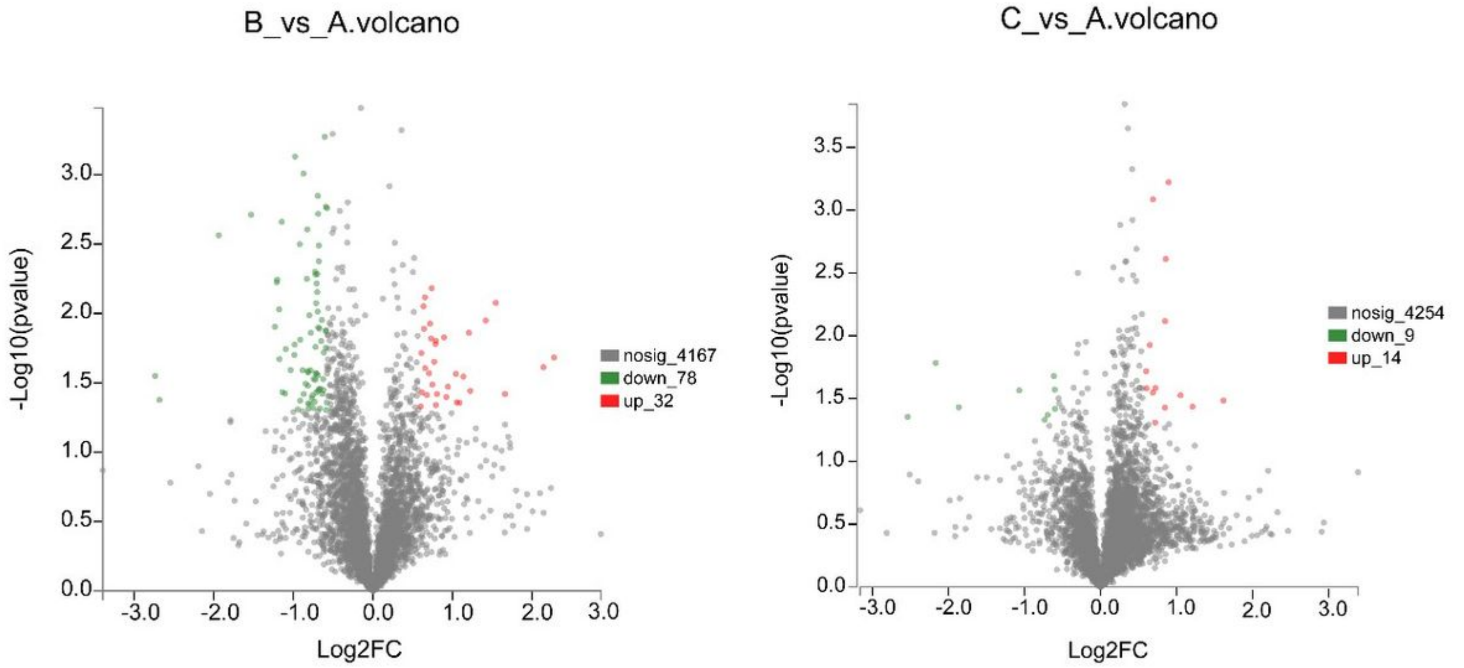
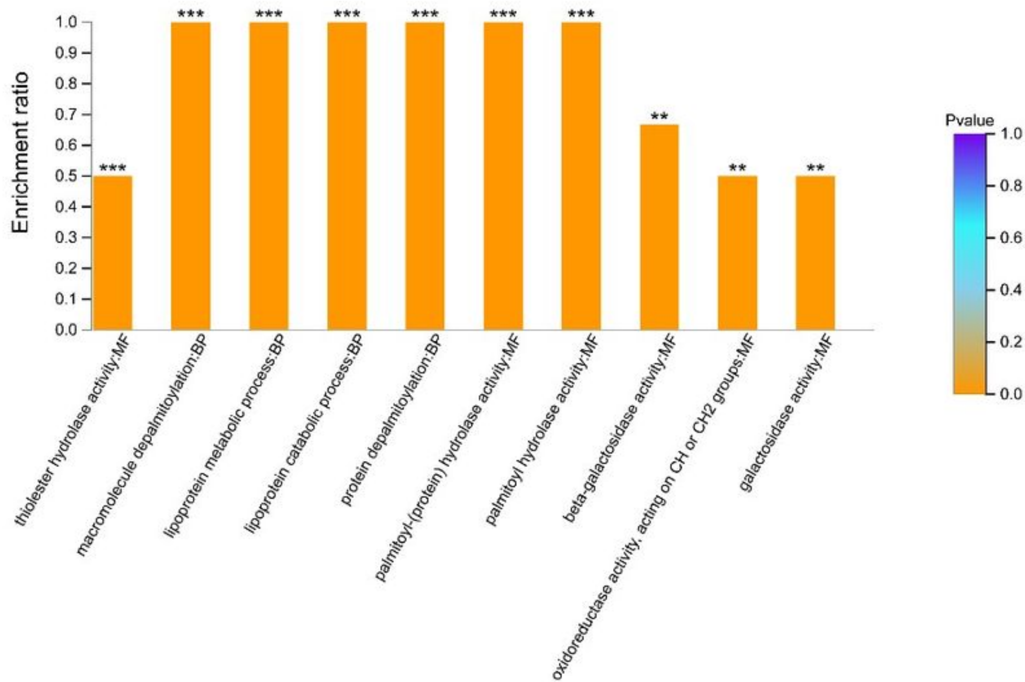


Figure 2

Volcano map of differentially expressed proteins between groups.



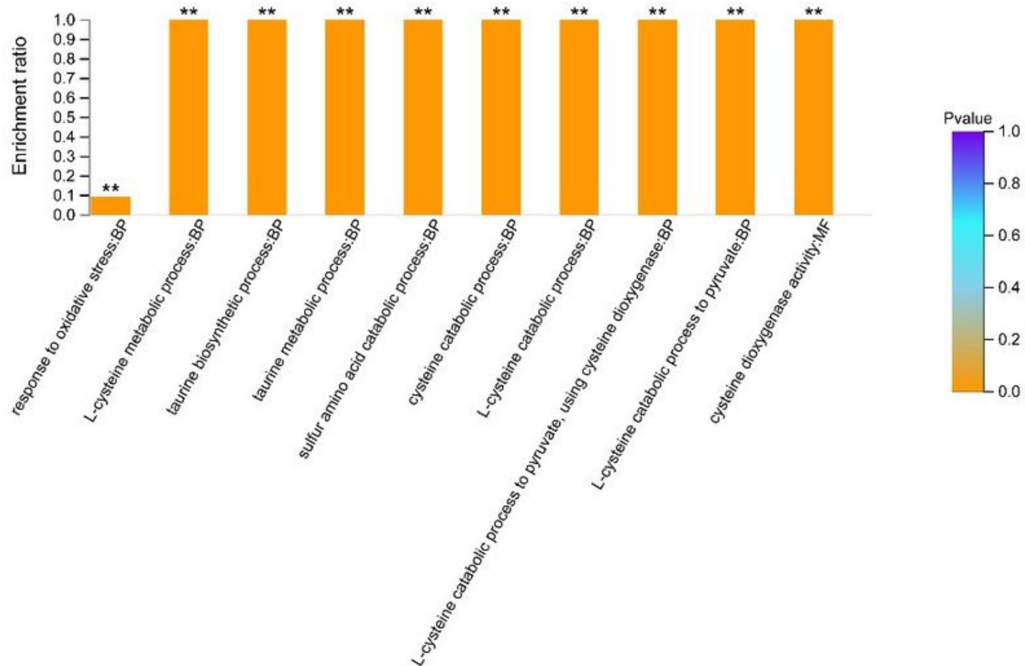
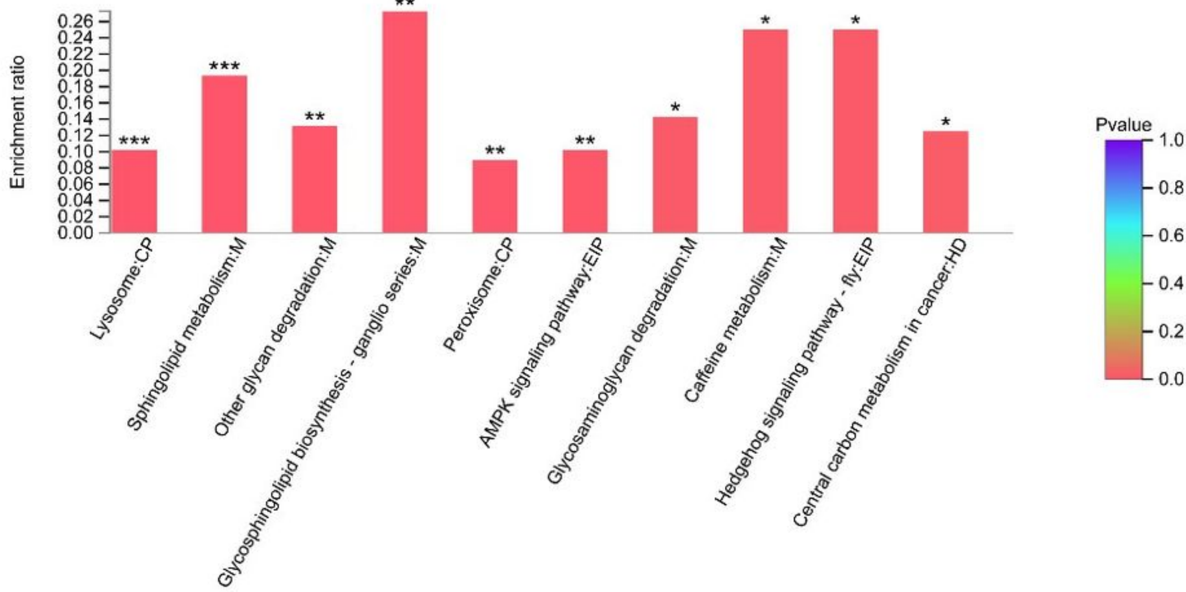


Figure 3

Gene ontology (GO) annotation of differential expressed proteins between groups. Note: *** P< 0.001, ** P < 0.01, * P < 0.05.



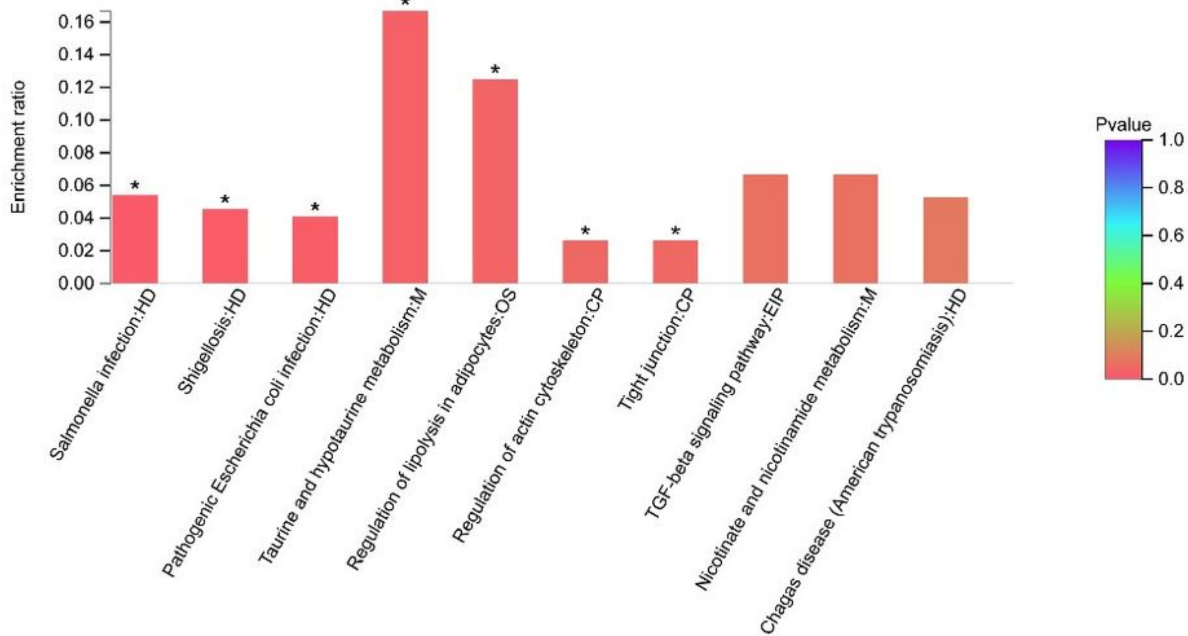


Figure 4

KEGG pathway enrichment analysis of differential proteins. Note: *** P< 0.001, ** P < 0.01, * P < 0.05.