



Dietary soy, pork and chicken proteins induce distinct nitrogen metabolism in rat liver

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ABSTRACT

Diets have been shown to alter metabolism and gene expression. However, few data are available about changes in gene expression in liver after intake of different meat protein diets. This work aimed to explore the long-term effects of protein source on liver metabolic enzymes. Rats were fed protein diets for 90 days to study whether intake of chicken and pork protein diets promoted gene expression involved in hepatic metabolism. Liver proteome profiles were measured by iTRAQ labeling and LC-ESI-MS/MS. Chicken protein diet induced higher level of serum amino acids in rats than soy protein. Amino acid metabolizing enzymes were downregulated by pork and chicken protein diets compared with soy protein diet. Intake of meat protein diets downregulated enzymes involved in protein synthesis, disulfide bond formation, signal peptide addition, transport, localization, degradation and glycosylation modification, but upregulated enzymes involved in prolyl *cis*-*trans* isomerization for protein synthesis. Protein diets from different sources affected the amino acid supply, and further influenced ribosome assembly and protein synthesis through mTOR signaling pathway.

1. Introduction

Humans and animals require an adequate supply of dietary protein. The digestion, absorption and metabolism of dietary proteins affect physiological functions, including weight maintenance, energy efficiency, appetite regulation and body composition (Jane et al., 2006; Verzola et al., 2020). Increasing numbers of studies have focused on the regulatory role of dietary protein in lipid metabolism and metabolic syndromes (Kang, Lee & Baik, 2011; Keller, 2011). However, previous studies have paid much attention to the effects of high protein intake (Heather & Leidy, 2014; Van et al., 2013) and dietary protein restriction (Deval et al., 2009) on physiological responses of the body. On the other hand, the functions of casein and soy protein in diets have been widely studied (Ascencio et al., 2004; Sousa et al., 2012), but the effect of meat protein has been studied very little.

Pork and chicken are the main meat source in Asia, but the advantages and disadvantages of meat protein have not been fully studied when compared with soy protein. Song et al. (2016) found that pork and chicken protein diets downregulated proteins involved in fatty acid metabolism and Ppar α signaling pathway. Another study also showed that pork and chicken protein diets differed from soy and casein protein

diets in biotransformation and antioxidation (Shi et al., 2018). However, the underlying mechanism was not fully understood.

The amino acid composition of protein diet may affect metabolism related gene expression. Volk et al. (2020) reported that amino acids of dietary protein may affect the body function by regulating the hormone level. Recent studies showed that dietary proteins and their amino acid composition were associated with the expression of genes involved in several metabolic pathways (Schwarz et al., 2012; Guo et al., 2019). In addition, mTOR signaling pathway was shown to be sensitive to amino acid abundance (Kim & Guan, 2011).

Taken together, meat is a good source for high quality protein. However, its amino acid composition differs greatly from plant protein, which affects liver metabolism. However, the underlying mechanism is still to be determined. In the present study, we compared the effects of pork or chicken protein diets with soy protein diet on liver proteomics and body function in rats, and the underlying mechanisms were discussed. The results would help give a good understanding of the associations between dietary protein and liver metabolism.

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2. Material and methods

2.1. Diets and animals

All animals were handled according to the guidelines of the Ethical Committee of Experimental Animal Center of Nanjing Agricultural University. Thirty-three 4-week-old male Sprague-Dawley rats (117 g \pm 10 g) were purchased from Zhejiang Experimental Animal Center (Hangzhou, Zhejiang, China) and raised in a specialized pathogen-free animal center. After one week of adaptation, the rats were assigned to one of three groups. The rats were fed with protein diets formulated with pork, chicken or soy protein diets for 90 days. The animals were kept individually in plastic cages, fed water and diets freely in a control room with temperature (20.0 \pm 0.5 °C) and humidity (60 \pm 10%). The light–dark cycle was 12 h.

Diets were prepared as described by Zhu et al. (2015). Pork *longissimus dorsi* muscle and chicken *pectoralis major* muscle were cooked to a central temperature of 70 °C in water bath for 0.5 h. Then, cooked meat was frozen, freeze-dried, and ground into powder. Intramuscular fat was removed with a mixture of solvent methylene chloride/methanol (v/v = 2:1). Soy protein was obtained from Linyi Shansong Biological Products Inc (Linyi, Shandong, China). The isoflavone was removed by alcohol extraction. Animal diets were prepared according to the formulation of the American Institute of Nutrition (AIN-93) to satisfy the nutritional needs for growing rats (Supplementary Table 1).

2.2. Quantitative proteomic analysis

2.2.1. Sample collection and protein preparation

After 90 days of feeding, the rats were fasted for 4 h and then killed by head dislocation. Liver samples were obtained, snap frozen in liquid nitrogen, and stored at –80 °C until analysis. Each protein diet group had 11 biological samples (n = 11).

Liver protein was extracted. Liver sample (0.1 g) was homogenized for 60 s at 7500 rpm by Precellys® Evolution (Bertin Technologies, Montigny-le-bretonneux, France). Then, the sample was centrifuged at 4 °C, 16,000g for 1 h. The supernatant was collected and mixed with 5 volumes of cold acetone containing 10% (v/v) TCA and kept at –20 °C for 4 h. The sample was centrifuged at 16,000g at 4 °C for 15 min and the supernatant was discarded. The pellet was washed in chilled acetone for three times, and then dissolved in urea buffer (8 M urea + 0.1 M HCl, pH 8.5), sonicated and centrifuged at 16,000g at 4 °C for 15 min. The supernatant was transferred to a new tube and protein concentration was quantified.

Appropriate amount of protein sample (150 μ g) was mixed with 400 μ L urea buffer in the ultra-filtration tube and centrifuged at 14,000g at 4 °C for 15 min. Two hundred microliters of urea buffer and 50 mM DTT were added to mix the samples, which were incubated at 60 °C for 1 h. The samples were mixed with 50 mM iodoacetamide to block the cysteine and then incubated at 25 °C for 45 min in dark. The supernatant was mixed with 100 μ L dissolution buffer (from iTRAQ kit). Then the mixture was centrifuged at 30,000g at 4 °C for 15 min and the supernatant was discarded. Purified protein was digested with trypsin at a ratio of 30 to 1 (protein to trypsin) at 37 °C for 16 h. Digested peptides were collected by centrifugation, and quantified using a NanoDrop spectrophotometer (ThermoFisher Scientific, Carlsbad, CA, USA).

2.2.2. iTRAQ labeling and high pH reverse phase fractionation

Peptides were labeled according to the manufacturer's protocol for 8-plex iTRAQ reagent (Applied Biosystems, Foster City, CA, USA). One unit of iTRAQ reagent was reconstituted in 24 μ L of isopropanol. Then the iTRAQ reagent was mixed with samples. The labeled peptides were incubated at room temperature for 2 h. The six labeled samples from different diet groups were pooled and dried by vacuum centrifugation. A total of 11 peptide mixtures were prepared.

The iTRAQ-labeled peptides were fractionated by the high pH

reverse phase fractionation chromatography. Firstly, the peptide mixtures were reconstituted in 100 μ L of buffer A (98% acetonitrile, 2% H₂O) and loaded onto an ACQUITY UPLC BEH C18 2.1 \times 100 mm column (1.7 μ m, Waters, Milford, MA, USA). The peptides were eluted at a flow rate of 0.2 mL/min using a gradient of 97% to 3% buffer A, and 3% to 97% buffer B (2% acetonitrile, 98% H₂O) for 60 min. Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled into 60 fractions. According to the differences in the fraction polarity (fraction collection time), these fractions were conflated into 8 samples to improve test efficiency and vacuum-dried.

2.2.3. Nano LC-MS/MS

The identification of samples was performed as below. Each of the fractions was dissolved in 0.1% formate and then centrifuged at 14,000g for 20 min. The supernatant containing 1.5 μ g peptides was loaded onto a column (Acclaim PepMap100 C18, 100 μ m \times 2 cm, 5 μ m, 100 Å, Thermo Scientific, Carlsbad, CA, USA), and eluted on an analytical column (Acclaim PepMap RSLC, C18, 75 μ m \times 10 cm, 3 μ m, 100 Å, Thermo Scientific, Carlsbad, CA, USA) by a gradient of buffer A (0.1% formate) from 97% to 3%, and buffer B (80% acetonitrile, 0.1% formate) from 3% to 97% at a flow rate of 300 nL/min over 160 min.

Data-dependent MS/MS was performed using an LTQ-Orbitrap XL mass spectrometer, equipped with a nano-electrospray ion source (Thermo Fisher Scientific, Carlsbad, CA, USA). The electrospray voltage applied was 2.2 kV. Peptides were detected in the Orbitrap at a resolution of 60,000. For MS scans, the m/z scan range was 300–1,600 Da. The top five ions were selected for MS/MS analysis if they exceeded a threshold of 5,000 counts and were at least doubly charged. The normalized collision energy for high collision dissociation was set to a value of 40%, and the resulting fragments were detected with 7,500 resolution in the Orbitrap. Every ion selected for fragmentation was excluded for 60 s by dynamic exclusion.

2.2.4. iTRAQ data analysis

Raw data were analyzed by Proteome Discoverer Software (version: 1.4, Thermo Fisher Scientific, Waltham, MA, USA). Protein identification was performed using Sequest HT engine against the UniprotKB *Rattus Norvegicus* database. Searching parameters were set as follows: trypsin was chosen as the enzyme with allowance at two missed cleavages; Gln \rightarrow pyro-Glu (N-term Q), oxidation (M), deamidated as the potential variable modifications, and carbamidomethyl (C), iTRAQ 8 plex (N-term), and iTRAQ 8 plex (K) as fixed modifications. A mass tolerance of 10 ppm was permitted for intact peptide mass and 0.02 Da for fragmented ions. Percolator algorithm was applied to estimate the false discovery rate based on q-value, and only peptides at the 99% confidence interval were counted as the identified protein. For protein quantification, one protein should contain at least two unique peptides. When the average of |Fold Change (FC)| \geq 2.0 in meat protein diet groups compared to the soy group, the protein was considered as a differential protein.

2.3. Analysis of amino acid in serum

After the rats were killed by head dislocation, blood samples were centrifuged at 4 °C, 1500g for 15 min. The supernatant (serum) were collected. Free amino acids were analyzed as below. Protein was removed from serum by the addition of sulfosalicylic acid. After centrifugation at 10,000g for 20 min at 4 °C, the free amino acids in the supernatants were analyzed by ion-exchange chromatography on an automatic amino acid analyzer (L-8900; Hitachi, Tokyo, Japan). The detected amino acids included: phosphoserine (P-Ser), taurine (Tau), phosphorylethanolamine (PEA), aspartic acid (Asp), threonine (Thr), serine (Ser), glutamin acid (Glu), α -aminoadipic acid (α -AAA), glycine (Gly), citrulline (Cit), alanine (Ala), α -aminobutyric acid (α -ABA), valine (Val), cysteine (Cys), methionine (Met), cystathionine (Cysthi),

isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), β -alanine (β -Ala), β -aminoisobutyric acid (β -AiBA), γ -aminobutyric acid (γ -ABA), ethanol amine (EOH₂NH), hydroxylysine (Hylys), ornithine (Orn), lysine (Lys), 1-methylhistidine (1Mehis), histidine (His), 3-methylhistidine (3Mehis), carnosine (Car), arginine (Arg), hydroxyproline (Hypro), and proline (Pro).

2.4. RT-PCR

Total RNA was extracted from liver samples using a commercial RNA extraction kit (No.9796, Takara, Dalian, China). The purity and quantity of total RNA were measured by a Nanodrop 2000 spectrophotometer at 260 and 280 nm. A total of 500 ng RNA was reversely transcribed into first-strand cDNA using Prime Script RT Master Mix Kit (No.RR036A, Takara, Dalian, China) according to the manufacturer's protocols. The RT-PCR reactions were run using SYBR Premix Ex Taq Kit (No.RR420A, TaKaRa, China) in QuantStudio™ 6 Flex Real-Time PCR System (Thermo Scientific, Waltham, MA, USA). Primers were designed according to the public database at the National Center for Biotechnology Information (NCBI) and were synthesized by Sangon Biotech Co., Ltd (Sangon, Shanghai, China). Primers used for RT-PCR were presented in Supplementary Table 2. The amplification was performed in a total volume of 20 μ L, containing 10 μ L of SYBR Premix Ex Taq, 0.4 μ L of each primer (10 μ M), 0.4 μ L of ROX Reference Dye II, 2 μ L of cDNA and 6.8 μ L of sterilized doubled-distilled water. The RT-PCR program was as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 34 s and 95 °C for 15 s, and holding at 60 °C for 1 min. Each sample was performed in triplicate. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method. GAPDH was applied as reference gene to determine mTOR, 4E-BP1 and p70S6K.

2.5. Immunoblot for mTOR expression

Western blot technique was used to determine mTOR expression. Liver tissues (100 mg) were lysed with lysis buffer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Whole protein was quantified with an enhanced BCA protein assay kit (Nanjing Jiancheng, Nanjing, China). The protein samples were mixed with loading buffer and denatured. Protein (40 μ g) was loaded onto a 10% SDS-PAGE. Electrophoresis was performed at 80 V for 1.5 h at 4 °C. Then the proteins were blotted by electrodiffusion for 1.5 h at 90 V on nitrocellulose membranes. Blotted membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 h and then incubated with rabbit polyclonal mTOR antibody (BS5527, Bioworld, Hinckley, UK) for 12 h at 4 °C. After being washed six times in TBST, the blotted membranes were incubated with goat anti-rabbit IgG (BS13278, Bioworld, Hinckley, UK) for 2 h. Proteins were detected with an Image Quant LAS 4000 (GE Healthcare Life Science, Chicago, IL, USA). The intensity of target protein genes was normalized against β -actin.

2.6. Bioinformatics and statistical analysis

In the present study, the protein expression matrix was generated with DataMerge2 and normalized, then obtained differential abundance proteins by *t* test. A multi-omics data analysis tool, OmicsBean (<http://www.omicsbean.cn>), which integrates Gene Ontology (GO) enrichment, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, were employed to analyze differentially abundant proteins. Means were compared by the Duncan's multiple comparison under the SAS system (version 9.2). The significance level was set if $p < 0.05$ for all statistical analyses.

3. Results and discussion

3.1. Dietary protein affected the amino acid composition in rat serum

The contents of dietary protein and various nutrients in each group were consistent (Supplementary Table 1). The only variable among diet groups was the source of protein. Although soy, chicken and pork protein were all whole proteins, there were great differences in amino acids, especially essential amino acids (Supplementary Table 3). Therefore, there should be differences in the level of digestion, absorption and metabolism after intake. The level of free amino acids in blood represented the overall operational level of amino acid metabolism, so we measured the level of nitrogen-containing small molecules such as serum amino acids.

In pork protein diet, threonine, valine, methionine, lysine, alanine, histidine, and total level of essential amino acids were significantly higher than those of soy protein diet, however, aspartic acid, serine, glutamate, cysteine, proline and levels of non-essential amino acids were lower (Fig. 1A, $p < 0.05$). After 90 days of feeding, feed intake and weight of rats fed pork protein diet were numerically greater than those fed soy protein diet, but the difference was not statistically significant (Table 1, Supplementary Table 4). The levels of serum amino acids in rats fed pork protein diet were lower than those in rats fed soy protein diet (except threonine) (Fig. 1A, $p < 0.05$).

In chicken protein diet group, the levels of threonine, valine, methionine, isoleucine, lysine, alanine and histidine were higher than those of soy protein diet group (Fig. 1B, $p < 0.05$). The total levels of branched-chain amino acids and essential amino acids were also higher than those in soy protein diet group, while the levels of phenylalanine, aspartic acid, serine, glutamic acid, cysteine and proline were lower (Fig. 1B, $p < 0.05$). The levels of serum amino acids, especially of threonine, valine and leucine were higher in chicken-protein-fed rats than those in soy-protein-fed rats ($p < 0.05$).

Dietary protein significantly altered serum metabolites in rats (Fig. 2). Serum metabolites in rats fed pork and chicken protein diets were more abundant than in rats fed soy protein diet. Pork and chicken protein diets significantly increased levels of serum α -aminobutyric acid, hydroxylysine, 1-methyl histidine, 3-methyl histidine, and histidine. α aminobutyric acid, β amino isobutyric acid and γ amino butyric acid were metabolites of isoleucine, glutamic acid, uracil, thymine and cytosine. 1-methylhistidine and 3-methylhistidine were post-translation modified amino acids. Serum 3-methylhistidine could serve as a reliable index for the breakdown of skeletal muscle protein in humans (Cruz et al., 2020).

3.2. Dietary protein changed the amino acid and nitrogen metabolism pathways in rat liver

3.2.1. Soy, pork and chicken proteins affected amino acid metabolism pathways

Proteome analysis indicated that dietary proteins regulated GSH, histidine, beta alanine, alanine, aspartic acid, glutamic acid, arginine and proline metabolism pathways and significant differences were observed between meat protein diet and soy protein diet groups (Fig. 3, $p < 0.05$).

In terms of phenylalanine and tyrosine metabolism, phenylalanine can be transformed into tyrosine by phenylalanine hydroxylase (monooxygenase), and tyrosine could be iodized into thyroxine (Sirmö et al., 2019). Lin et al. (2016) found that the free thyroid T3 and T4 in rats fed chicken and pork protein diets were significantly higher than in those fed soy protein diet, indicating that the iodization of phenylalanine and tyrosine were significantly higher in rats fed meat protein diets. In the body, tyrosine can be transformed into dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase, which can be further decarboxylated to produce dopamine, norepinephrine, epinephrine, and melanin (indole quinone polymer) (Singh et al., 2020). In terms of catabolism, tyrosine is

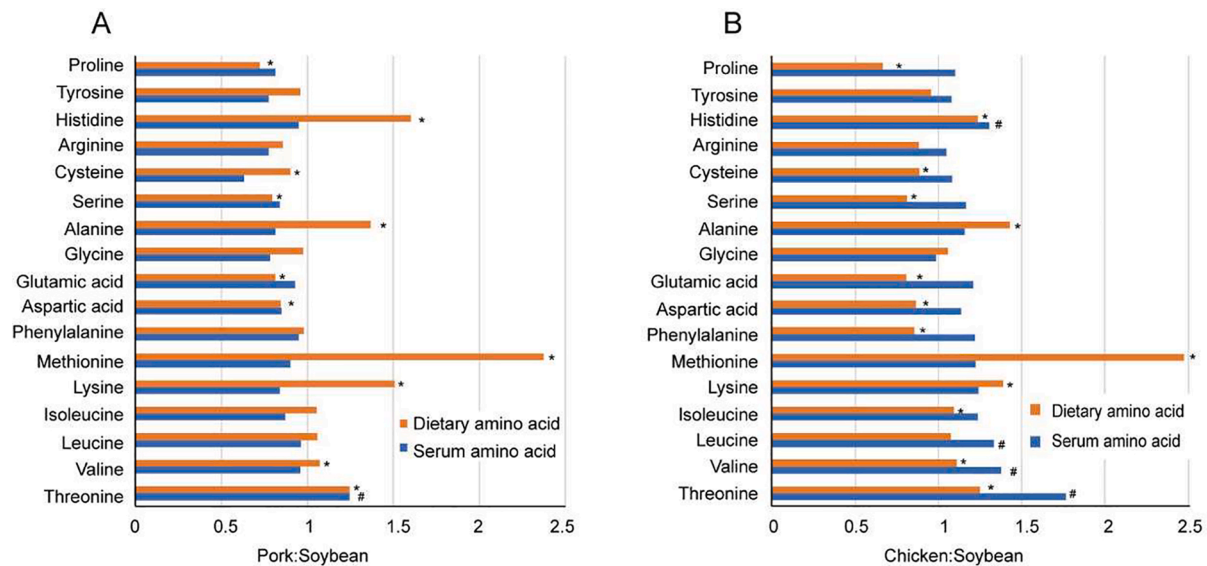


Fig. 1. Dietary and serum amino acid concentrations in rats fed pork or chicken protein compared with soy protein. Values are represented as the means \pm SD. Groups were compared by one-way ANOVA followed by Duncan's multiple range test. *, # represent significant differences between diet groups ($p < 0.05$).

Table 1

Effect of different proteins diets on body weight in rats ($n = 11$).

Weeks	Soy (g)	Pork (g)	Chicken (g)
0	167.60 \pm 12.32 ^a	167.82 \pm 14.74 ^a	167.10 \pm 11.18 ^a
1	243.40 \pm 15.72 ^a	263.27 \pm 23.29 ^a	261.00 \pm 14.12 ^a
2	297.60 \pm 19.52 ^b	315.80 \pm 17.29 ^{ab}	325.50 \pm 7.91 ^a
3	365.78 \pm 18.18 ^b	387.60 \pm 16.91 ^{ab}	392.40 \pm 24.36 ^a
4	406.22 \pm 18.88 ^b	420.00 \pm 21.85 ^{ab}	430.20 \pm 26.72 ^a
5	443.11 \pm 25.94 ^b	463.00 \pm 21.53 ^{ab}	470.73 \pm 35.06 ^a
6	493.33 \pm 32.70 ^b	508.91 \pm 26.43 ^{ab}	522.91 \pm 41.13 ^a
7	518.20 \pm 48.06 ^b	536.40 \pm 20.19 ^a	556.55 \pm 47.42 ^a
8	528.67 \pm 72.16 ^b	566.55 \pm 26.35 ^{ab}	581.09 \pm 56.27 ^a
9	554.40 \pm 67.71 ^b	586.18 \pm 25.84 ^{ab}	598.00 \pm 59.26 ^a
10	571.27 \pm 65.94 ^a	602.91 \pm 36.50 ^a	609.18 \pm 60.10 ^a
11	587.64 \pm 66.31 ^a	621.64 \pm 43.86 ^a	623.82 \pm 60.43 ^a
12	615.20 \pm 59.76 ^a	630.36 \pm 43.89 ^a	639.27 \pm 74.72 ^a
13	630.20 \pm 61.69 ^a	644.82 \pm 41.28 ^a	650.91 \pm 76.47 ^a
ADG(g/d)	5.19 \pm 0.68 ^a	5.26 \pm 0.29 ^a	5.38 \pm 0.39 ^a

Notes: ADG, average daily weight gain; a, b, different superscripts in the same row showed significant difference ($p < 0.05$).

decomposed into fumarate and acetoacetate, which participates in sugar and fat metabolism (Kaushal et al., 2020). The abundance of hydroxyphenylpyruvate dioxygenase (Hpd), phenylalanine-4-hydroxylase (Pah) and maleoyl acetoacetate isomerase (Gstz1) which catalyzes the catabolism of fumarate and acetoacetate, was significantly lower in rats fed chicken and pork protein diets than in rats fed soy protein diet (Fig. 3). This indicates that more phenylalanine may be catabolized in rats fed soy protein diet, while it could involve hormone anabolism in rats fed chicken and pork protein diets.

In terms of histidine metabolism, cytoplasmic non-intentional dipeptidase (Cndp2), which catalyzes the degradation of peptides to produce histidine, was less abundant in rats fed chicken and pork protein diets (Fig. 3). This indicates lower histidine catabolism in these two groups. Histidine has multiple metabolic transformation pathways in the body, which may be transformed into histidine trimethyl, thiohistidine methyl, and n-formyl-l-glutamate by histidine ammonia-lyase (Hal). N-formyl-l-glutamate can be further transformed into glutamate by formimino transferase cyclodeaminase (Ftcd) (Xia et al., 2020). As shown in Fig. 3, the abundance of Hal and Ftcd was lower in rats fed chicken and pork protein diets ($p < 0.05$). The relatively high histidine degradation caused lower serum histidine in rats fed soy protein diet. The histidine catabolism may link to lipid synthesis, which may explain that

rats fed soy protein diet weighed less but had a higher fat content in our previous study (Lin et al., 2016).

In terms of proline metabolism, the proline level in chicken and pork protein diet groups was significantly lower than that in soy protein diet group, but the proline level in serum increased greatly. This could be related to high expression of pyrrolic acid reductase 3 (Pycrl) in rats fed pork and chicken protein diets (Fig. 3), which catalyzes the synthesis of L-proline from glutamic semialdehyde in liver.

β -Alanine is an important product of cytosine and uracil catabolism. Beta-ureidopropionase (Upb1) catalyzes the decomposition of cytosine and uracil to β -alanine, ammonia and carbon dioxide (Penttilä et al., 2012). In the present study, the abundance of Upb1 was significantly lower in rats fed pork and chicken diets (Fig. 3).

Leucine is an important ketogenic and essential amino acid. Leucine can be transformed into 3-methylbutyryl coenzyme A by the deamination reaction, then into 3-methylbutene-2-enoyl coenzyme A by isovaleryl coenzyme A dehydrogenase (Ivd), and finally into 3-hydroxy-3-methylpentadiene coenzyme A (HMG-CoA) by methylcrotonyl coenzyme A carboxylase 2 (Mccc2) (Arnedo et al., 2019). HMG-CoA is an important intermediate product for the synthesis of cholesterol and ketones. It can be decomposed into acetoacetic acid and acetyl coenzyme A by 3-hydroxy-3-methylglutaryl coenzyme A lyase (Hmgcl). Acetyl coenzyme A could also generate acetoacetyl coenzyme A by acetyl coenzyme A acetyltransferase (ACAT1). Acetyl coenzyme A and acetoacetyl coenzyme A could generate HMG-CoA by hydroxymethylpentadiene coenzyme A synthase (Hmgcs2). In this process, HMG-CoA reductase (HMGR), the rate limiting enzyme for cholesterol synthesis, was not detectable. The abundance of Hmgc1, ACAT1, Hmgcs2, Ivd and Mccc2 was significantly lower in rats fed pork and chicken protein diets than in rats fed soy protein diet (Fig. 3). And thus leucine catabolism in liver was significantly lower in rats fed meat protein diets.

Urea cycle is an important form of ammonia metabolism in liver. Ammonia can be produced by deamination of amino acids starting with carbamoyl-p and transformed into urea through ornithine, citrulline, L-arginosuccinate and arginine (Mazi et al., 2019). In the present study, ornithine carbamoyl transferase (Otc), arginyl succinate synthase (Ass1) and arginyl succinate lyase (Asl) in urea cycle were less abundant in rats fed chicken and pork protein diets (Fig. 3), indicating a weaker catabolism of amino acids and deamination reaction in rats fed chicken and pork protein diets. In addition, arginine and ornithine could be changed into spermine and putrescine through decarboxylation reaction. Here,

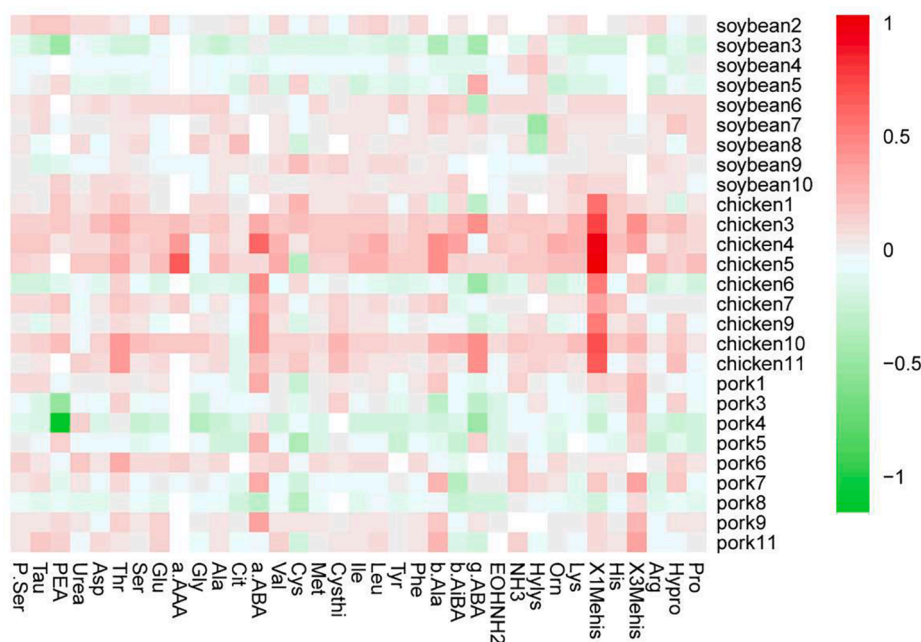


Fig. 2. Heatmap of serum amino acid concentrations of the rats fed pork, chicken and soy proteins.

spermidine synthase (Srm) catalyzing the reaction, was downregulated in rats fed pork protein diet (Fig. 3). Acyl amino acid hydrolase (Acy1), catalyzing the transformation of n-acetylmethionine into ornithine (Kurbatova et al, 2020), was also downregulated in rats fed pork protein diet (Fig. 3). These results indicate that pork protein diet may downregulate the urea metabolism pathways.

Enoyl-CoA hydratase (Ech) and hydroxyalkyl CoA dehydrogenase (Hadh) that are involved in leucine and β -alanine metabolism and β -oxidation of fatty acids, were significantly downregulated by pork and chicken protein diets (Fig. 3). In addition, α -amino adipic semialdehyde dehydrogenase (Aldh7a1) and trimethylamino butyraldehyde dehydrogenase (Aldh9a1), which catalyzes dehydrogenation of aldehydes and semialdehydes to produce acids, were also downregulated by pork and chicken protein diets (Fig. 3). Endogenous amino acids and amines could be oxidized into aldehydes, which were further irreversibly oxidized into acids by aldehyde dehydrogenase (Luo et al., 2014). This indicates that meat protein diet could reduce the accumulation of aldehydes.

3.2.2. Soy, pork and chicken protein affected nitrogen metabolism pathways

Intake of different protein diets leads to differences in digestion and absorption, and finally affects metabolism in liver. The difference in amino acid composition was the only factor for changing many biological processes. Blood is a channel for amino acid transport to different organs of the body. The concentration of amino acids in blood is affected by nutritional and pathological conditions.

By comparing the levels of amino acids in diet and rat serum, it was found that chicken protein diet significantly increased the blood amino acid level. Pork protein had relatively high levels of essential amino acids than soy protein, but the contents of essential amino acids in serum of rats fed by pork and soy protein diets were similar. Lin et al. (2016) found that the free T3 and T4 in rats fed pork protein diet were higher than those in rats fed soy protein diet, indicating that meat protein diet could increase energy consumption and reduce anabolism. Pork contains higher ketogenic leucine and lysine, which increased the supply of ketone oxidation without participating in adipogenesis. Meanwhile, alanine was high in both chicken and pork proteins, which could be deaminated to pyruvate and produce glucose through gluconeogenesis.

Amino acids were related to sugar and nucleotide metabolism through carbon chain and nitrogen metabolism.

Previous studies have focused on the differences of lipid metabolism caused by diets, but few studies explained the causes for metabolic differences by the level of dietary amino acids. Song et al. (2016) have studied short-term effects of different protein diets. In the present study, pork protein diet was also shown to inhibit the metabolism of valine, leucine, isoleucine, proline, alanine, aspartic acid and glutamate. In addition, meat protein diets downregulated gene expression involved in metabolism of essential amino acids but upregulated gene expression related to amino acid transport in both short-term and long-term studies. In this study, chicken protein diet did not improve the TCA cycle, but soy protein diet promoted amino acid catabolism, resulting in the transformation of amino acids into sugar and lipid, which explains the weight differences of rats in the two studies.

3.3. Dietary protein changed transcription, translation and intracellular transport and metabolism of protein in liver

Dietary protein source significantly affected the abundance of proteins in rat liver involved in mRNA transcription, ribosome assembly, translation, modification, and protein degradation (Fig. 4). Histones are basic proteins, mainly comprising of arginine and lysine. Meat contains higher lysine but lower arginine than soy. In the present study, histones Hist1h4b and Hist2h2ac were downregulated by chicken and pork protein diets, while histone Hist1h1c was upregulated by the same diets. Compared with soy protein diet group, the nuclear export protein (Alyref) and the mRNA decay and unwinding protein (Dhx9) were upregulated by chicken and pork protein diets. Several proteins related to RNA transcription were also upregulated by meat protein diets, suggesting that meat protein diets had a positively regulatory effect on transcription.

In terms of ribosomal proteins, the abundance of 40S and 60S ribosomal subunit related proteins (Rps25, Rps13, Rps17) was higher in rats fed chicken and pork protein diets than in rats fed soy protein diet, indicating that meat protein diets may promote protein synthesis in liver more efficiently.

Protein disulfide isomerase is an important allosteric enzyme in the process of endoplasmic reticulum protein synthesis (Tang et al., 2019).

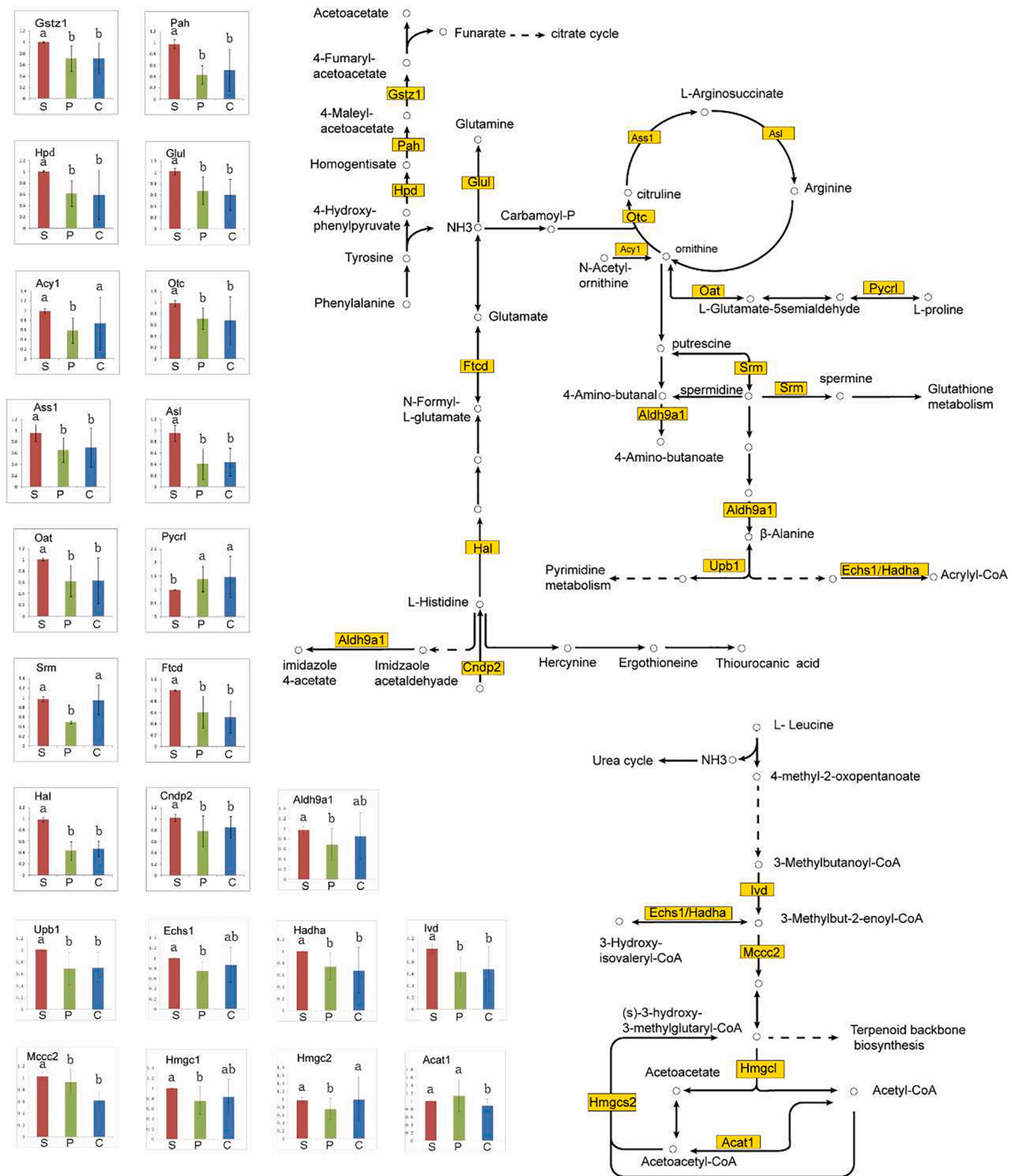


Fig. 3. Effect of dietary soy, pork and chicken protein on amino acid metabolic pathways. S: soy; P: pork; C: chicken. a, b represent significant differences between diet groups ($p < 0.05$).

This enzyme catalyzes the breaking of mismatched disulfide bonds. In the present study, protein disulfide isomerase A6 (Pdia6) was down-regulated by chicken and pork protein diets, indicating that less protein assembly of disulfide bonds occurred in liver of rats fed meat protein diets.

In protein synthesis, proline can form cis and trans peptide bonds. In natural proteins, peptidyl proline was mostly in trans configuration, and only 6% was in cis configuration (Joseph et al., 2012). Peptidyl proline cis trans isomerase (Ppi) catalyzes accurate folding of the polypeptide chain. In the present study, several peptidyl proline cis trans isomerases (Ppib, Fkbp2, Fkbp3) were highly expressed in rats fed pork and chicken

protein diets, indicating that meat protein diets could promote the proline conformational changes, correct folding and assembly, degradation and reuse.

In cells, newly synthesized proteins undergo post-translational modifications. Dietary protein has been shown to be an amino acid donor for protein synthesis in liver tissue (Madrigal et al., 2020). The present study showed significant differences in proteins involved in protein synthesis and modification. The enzymes catalyzing the degradation of cell proteins in lysosomes and proteasomes were significantly lower in rats fed meat proteins than in rats fed soy protein. Our previous study showed that short-term intake of pork protein diet upregulated

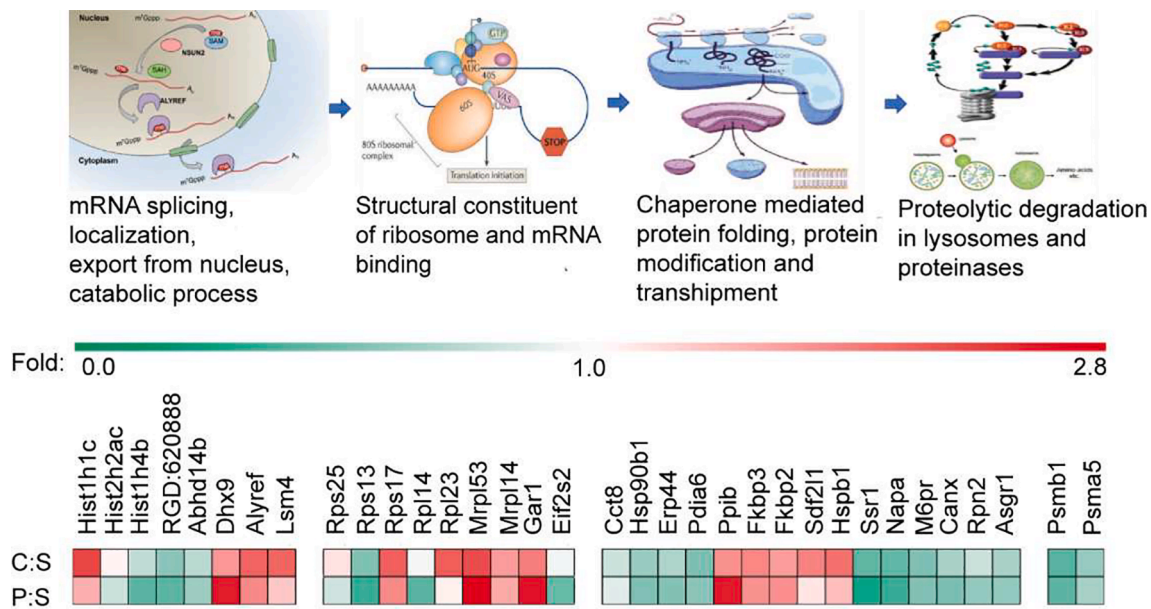


Fig. 4. Effect of pork and chicken protein dietary on transcription, translation, and protein transport, and metabolism in rat liver. C:S, chicken:soy; P:S, pork:soy.

protein expression in rat liver related to protein biosynthesis (translation, mRNA processing and tRNA aminoacylation) (Song et al., 2016). The differences between the two studies may be due to the different protein synthesis and amino acid utilization.

3.4. Effects of dietary protein on mTOR pathway

There was no significant difference in mTOR mRNA abundance in rats among diet groups (Fig. 5C). However, western blotting showed that the mTOR protein was higher in rats fed chicken protein diet than that of rats fed soy protein diet (Fig. 5D). The mRNA level of its downstream protein 4EBP1 was different between the two meat protein diet groups

(Fig. 5A). The mRNA level of another downstream kinase p70S6K was downregulated by chicken and pork protein diets compared with soy protein diet group (Fig. 5B).

The mTOR is a highly conserved serine and threonine kinase, comprising of rapamycin and nutrient sensitive mTOR complex 1 (mTORC1) and the regulatory associated protein of mTOR (rapTOR) (Crino, 2011). Previous cell studies showed that amino acids can activate p70S6K and 4EBP1 through mTOR signaling pathway, and promote protein translation (Hara et al., 1998). In the absence of amino acids, p70S6K will be rapidly inactivated, and 4EBP1 will be dephosphorylated. As a result, protein translation will be stopped. Leucine, arginine, glutamine, glutamic acid, and proline are involved in the activation of

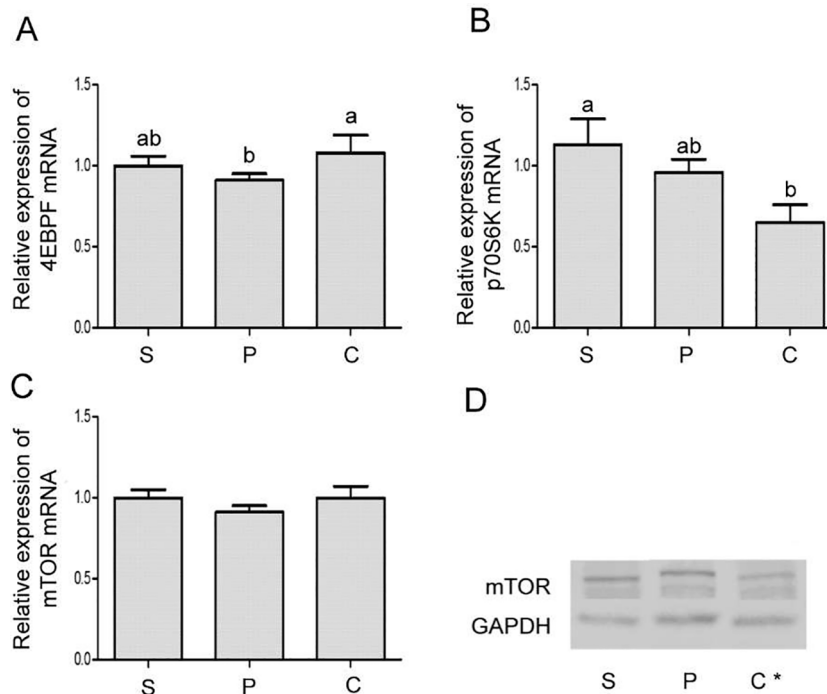


Fig. 5. Effect of pork, chicken and soy protein dietary on mTOR pathway related genes or protein (western blot) in rat liver. Values are represented as the means \pm SD. Means were compared by one-way ANOVA and Duncan's multiple range tests. a,b represent significant differences between diet groups ($p < 0.05$).

mTOR (Durán et al., 2012; Han et al., 2012; Wolfson et al., 2016).

Kimball and Jefferson (2006) observed that leucine promoted overall mRNA translation and stimulated protein synthesis. The key proteins of the regulation mechanism were that p70S6K and 4EBP1. In this study, there was no significant difference in leucine in raw protein, but significant differences were observed in serum leucine of rats among pork, chicken and soy protein groups. Such a difference provides a material basis for the different activity of mTOR pathway. Therefore, the impact of diet on mTOR pathway should be attributed to multiple amino acids.

4. Conclusion

Liver is one of the most important organs in human body for metabolism. The effect of dietary meat and soy protein diets on body health can be reflected by nitrogen metabolism and related biochemical changes in liver. In this study, we investigated the proteomic profiles in rat liver after 90 days feeding chicken, pork and soy protein diets, focusing on nitrogen metabolism. Amino acid metabolizing enzymes were downregulated by pork and chicken protein diets compared with soy protein diet. Intake of meat protein diets also downregulated enzymes involved in protein synthesis, disulfide bond formation, signal peptide addition, transport, localization, degradation and glycosylation modification, but upregulated enzymes involved in prolyl cis-trans isomerization for protein synthesis. Protein source affected the amino acid supply, and further ribosome assembly and protein synthesis through mTOR signaling pathway. The findings provide an insight into the underlying mechanism on the regulatory role of dietary protein in liver metabolism.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochms.2021.100050>.

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