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RESEARCH ARTICLE

Proteome comparisons reveal influence of different dietary proteins on the development of rat jejunum

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Abstract This study compared proteome profiles and morphological changes of rat jejunum in response to different dietary proteins. Fifty male Sprague-Dawley rats were fed with casein (control), and isolated beef, pork, fish and chicken proteins for 14 days. Proteome analysis, histological observation and PEPT1 quantification of the jejunum were performed. The results indicated that rats fed with chicken proteins had higher PEPT1 mRNA and protein levels ($P < 0.05$) but lower villus height and ratio of villus height to crypt depth (V/C ratio, $P < 0.05$) than those fed with casein and pork protein. Label-free LC-MS/MS indicated that, as compared to casein, intake of chicken protein can regulate oligopeptide transport mainly by upregulating PEPT1 protein expression and reducing dipeptidyl-peptidase activity related to biological oxidation, and can reduce oligopeptide absorption capacity by regulating Hippo signaling pathway. Although intake of beef and fish proteins had no significant effect on PEPT1 expression, they altered several signaling pathways.

Keywords Hippo signaling pathway, meat protein, PEPT1, proteome analysis, rat jejunum

1 Introduction

Dietary protein affects the intestinal morphology and amino acid transport. Previous studies indicated that a high protein diet increased amino acid uptake in the jejunum, while a protein-deficient diet reduced uptake of non-essential amino acids but increased uptake of essential amino acids and alanine[1]. Kuhla et al.[2] observed that partial substitution of milk protein for soy protein increased the V/C ratio of goat jejunum and changed the proteome profile of jejunum involving protein turnover, energy metabolism and cytoskeleton assembly, and those authors attributed the differences to the composition of essential amino acids . Numerous studies have focused on amino acids nutrition in the field of farm animal science[3–6]. For example, glutamine supplementation can increase enterocyte proliferation[3], but arginine supplementation may cause lower cell proliferation and greater enterocyte apoptosis[4]. Tryptophan and L-leucine were also shown to have a significant effect on intestinal morphology and tight junction proteins[5,6].

Dietary proteins are digested and absorbed in the gastrointestinal tract. Peptide transporter PEPT1/SLC15A1 plays a critical role in uptake of dipeptides and tripeptides from the lumen to the cells[7]. PEPT1 belongs to proton-coupled oligopeptide transporter superfamily, and is mainly located in the brush-border membrane of enterocytes[8,9], and villus height and crypt depth of enterocytes reflect the gut absorption capacity[10,11]. Nutrient absorption is related to many aspects, in particular to the abundance of transmembrane transporters, energy metabolic enzymes and regulatory factors[12]. PEPT1 expression may be in turn regulated by the level and origin of dietary proteins[13–15].

Nutritional values differ between plant and meat proteins[16,17], because of different protein composition (especially the essential amino acid profiles) and digestibility[18]. Given that oligopeptide transporters play a more critical role in the uptake of digested proteins than amino acid transporters[19], different protein sources might be expected to induce changes in PEPT1 expression. A previous study examined the effects of soy protein and four meat proteins on morphology and PEPT1 expression of rat duodenum and jejunum after 7 days feeding, and showed that beef, pork, chicken and fish protein in diets potentially have different effects on animal gut development[20]. However, few data or information about underlying mechanisms are available about the differences between these dietary proteins and implications for long-term feeding.

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This study aims to compare the differences in PEPT1, morphology, and proteome profiles in rat jejunum after feeding with casein (control), beef, pork, chicken and fish proteins for 14 days. Bioinformatics analysis revealed information about the possible underlying mechanism.

2 Materials and methods

2.1 Diets and sampling

2.1.1 Diets

Diets for growing rats were prepared according to the AIN-93G diet formulation[21], with replacement of casein by the same quantity of isolated proteins from beef, pork, chicken or fish according to the treatment. Casein extract was sourced from Jiangsu Xietong, Inc. (Nanjing, China). Meat proteins were extracted as previously described[22] with minor modifications. Briefly, pork and beef *longissimus dorsi* muscles, chicken *pectoralis major* muscle and fish (carp) dorsal muscle were cut, packed and cooked in a water bath at 70°C. Then the cooked samples were ground and fats removed twice by methylene chloride/methanol (2:1, v:v). The residual solvent in the powder was fully evaporated in a fume hood for 24 h. The meat protein powders were sieved through a 25 mesh and the resulting powder contained greater than 92% protein and 6%–8% water. Diets were prepared by Jiangsu Xietong, Inc. (Nanjing, China). Details of these diets, including ingredient composition, have been reported by Song et al.[23]. The amino acid profiles in the diets are shown in the Table S1.

2.1.2 Animals and sampling

The animal experimental protocol was approved by the Ethics Committee of the Experimental Animal Center of Nanjing Agricultural University (license number SCXK (Su) 2002-0029). Fifty male Sprague-Dawley rats (3 weeks old) were obtained from Shanghai Laboratory Animal Research Center (Shanghai, China). Animals were housed in pairs at 23±2°C and with a 12 h light/dark cycle. To adapt to new conditions, all rats received the AIN-93G diet that contains casein for 7 days. After that, animals were divided into five diet groups (10 rats per group), i.e., AIN-93G (casein), beef protein, pork protein, chicken protein and fish protein. The animals were provided with diets and water *ad libitum*. After 14 days feeding (animal age: 42 days old), the rats were killed by cervical dislocation after 4 h deprival of diets. The jejunum was taken out and immediately rinsed with 0.9% NaCl solution. Segments (1 cm) of the proximal jejunum were collected and fixed in 10% formalin buffer for 12 h for histological observations. The remainder of the jejunum was used for qRT-PCR, western blotting and proteomic analyses.

2.2 Quantification of PEPT1

2.2.1 qRT-PCR

The TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Ostu, Japan) was used for total RNA extraction of jejunal samples according to the manufacturer's instructions. Total RNA was quantified by a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, DE, USA). The RNA was reverse transcribed to cDNA using PrimeScript™ RT Master Mix (TaKaRa, Ostu, Japan) in a Peltier Thermal Cycler 200 (MJ Research, Watertown, MA, USA). The cDNA was dissolved in RNase-free water and stored at −20°C. The two-step qRT-PCR reactions were performed in triplicate on 96-well plates using a 7500 Real-time PCR system (Applied Biosystems, Foster, CA, CA) with the SYBR® Premix Ex Taq™ (TaKaRa, Ostu, Japan). The *Pept1* primers were designed using the Primer 3.0 (Applied Biosystems), and the *Gapdh* primers (reference gene) were provided by Sangon Biotech (Shanghai, China) (Table 1). The concentrations of template, primers and amplified products were evaluated by relative standard curves made by multiple dilution (1:1 to 1:625). The reaction solution (20 µL) contained 10 µL SYBR® Premix Ex Taq, 0.4 µL PCR forward primer (10 $\mu mol \cdot L^{-1}$), 0.4 µL PCR reverse primer (10 $\mu mol \cdot L^{-1}$), 0.4 µL ROX reference dye II, 2 µL cDNA and 6.8 µL dH_2O. Cycling conditions were as follows: 30 s for denaturation at 95°C, 40 cycles of 5 s at 95°C and 34 s at 60°C for denaturation, followed by three alternations between 95°C and 60°C for melting curve analysis to verify the specificity of a single amplification. Fold changes of *Pept1* expression were calculated by the $2^{-\Delta\Delta C_T}$ method[24] normalized to *Gapdh*, and casein group was set as the control.

Table 1 Primers used for qRT-PCR

Gene	Primer	Sequence (5′–3′)	Length/bp	Product size/bp	Tm/°C	GenBank Accession no.
Pept1	Forward	GTA TGT TCT GTT CGC CTC CTT G	22	228	60.07	NC_005114.3
	Reverse	GGT GAA TGC TGG ACT TGG TAT G	22		60.07	
Gapdh	Forward	ACA GCA ACA GGG TGG TGG AC	20	252	57.70	NC_005103.4
	Reverse	TTT GAG GGT GCA GCG AAC TT	20		57.80	

2.2.2 Western blotting

The mucosa was separated from jejunal samples and proteins were isolated from epithelial cells with a commercial protein extraction kit (Beyotime, Nantong, China) according to the manufacturer's instructions. To avoid any enzymatic reactions during preparation, two enzyme inhibitors (Sigma-Aldrich, St. Louis, MO, USA), i.e., protease inhibitor complete (one tablet per 50 mL) and phosphatase inhibitor phosStop (two tablets per 50 mL) were added into cold protein extraction buffers. Protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL, USA). The protein extracts were lysed in 4 × protein loading buffer (Solarbio, Beijing, China) and heated for 5 min at 80°C. Proteins (30 μg) were separated on 10% SDS polyacrylamide gels (Bio-Rad, Laboratories, Hercules, CA, USA) electrophoresed at 100 V for 1h, and then transferred onto a polyvinylidene fluoride (PVDF) membrane at 90 V for 90 min at 4°C. Transfer buffer contained 10% (v/v) 10 × Tris/Glycine buffer (Bio-Rad, Laboratories), 15% (v/v) methanol and 0.01% (w/v) SDS. After 2 h incubation in chemiluminescent blocker (Millipore, Temecula, CA), the membranes were rinsed in TBST [10% (v/v) 10 × TBS buffer (Bio-Rad, Laboratories), 0.1% (v/v) Tween-20] and incubated with the primary antibodies against PEPT1 (1:500; Bioworld) and β-actin (1:3000; Bioworld) overnight at 4°C. The blots were washed for 30 min in TBST and probed with the HRP-labeled secondary antibody (1:5000; Bioworld) for 1.5 h at room temperature and then washed again. Proteins were visualized using a chemical luminescence imaging analyzer (ImageQuan LAS 400; GE Healthcare Life Sciences, Pittsburgh, PA, USA) after being incubated with ECL western blotting substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA). Band intensities were quantified using Quantity One software (Bio-Rad Laboratories). PEPT1 expression was normalized to β-actin.

2.3 Proteome analysis

2.3.1 Sample preparation

Proteins were isolated from epithelial cells of jejunal mucosa as above described, then re-suspended in 8 mol·L^{-1} urea and precipitated by adding three volumes of pre-cooled acetone. The samples were incubated at −20°C for 12 h and then centrifuged at 14000 r·min^{-1} for 15 min. The pellet was washed twice with the same volume of pre-cooled acetone, centrifuged for 5 min, resuspended in 8 mol·L^{-1} urea and then reduced with 20 mmol·L^{-1} DTT at 60°C for 1 h. Proteins were then alkylated in the dark with 40 mmol·L^{-1} iodoacetamide at 22°C for 30 min. The alkylation reaction was quenched by 10 mmol·L^{-1} DTT and samples were diluted to 2 mol·L^{-1} urea with HPLC grade water. Protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL, USA). An appropriate amount of trypsin was mixed with the sample at an enzyme-to-substrate ratio of 1:100, and tryptic digestion was performed at 37°C for 18 h in 100 mmol·L^{-1} triethylammonium bicarbonate (pH 8; Sigma-Aldrich). The digested products were desalted with ZipTip (Millipore, Temecula, CA, USA) and freeze-dried for LC-MS/MS analysis.

2.3.2 LC-MS/MS analysis

Dried trypsin-digested products were dissolved in 12 μL of 0.1% FA, and identified by a nano-LC system (Eksigentekspert™ nanoLC 425; AB Sciex, Framingham, MA, USA) coupled to an AB Sciex Triple TOF® 6600 system (AB Sciex). Peptides were trapped in a Chrom XP nanoLC C18 trap column (350 μm id × 0.5 mm long, 3 μm, 120Å), and eluted at a flow rate of 300 nL·min^{-1} into a reverse-phase C18 column (75 μm id × 15 cm long, 3 μm, 120Å). A linear gradient elution was applied using 3% to 36% acetonitrile in 0.1% formic acid with a total running time of 120 min. Mass spectra were recorded in positive-ion and high-sensitivity mode with a resolution of 35000 full-width half-maximum. The nanospray needle voltage was 2300 V. For collision induced dissociation tandem mass spectrometry (CID MS/MS), the mass window for precursor ion selection of the quadrupole mass analyzer was set to ±2 m/z. The precursor ions were fragmented in a collision cell using nitrogen as the collision gas. Advanced information dependent acquisition (IDA) was used to obtain MS/MS spectra.

2.3.3 Mass data analysis

The MS generated files were searched against the UniProt, NCBI and common MS contaminant databases using Mascot 2.5 (Matrix Science, Boston, MA, USA) software. The tolerance for MS1 and MS2 error was set to 50 mg·kg^{-1} and 50 Da, respectively. A maximum of 2 trypsin miss cleavages were allowed. Peptides were assumed to have a charge of 2 + , 3 + or 4 +. The mass input was assumed to be mono-isotopic mass. Decoy database was used to control the false discovery rate (FDR) less than 1%. The search result was exported to CSV file for label-free quantification using in-house software.

Label-free quantification was performed by using normalized spectral index (SI_n) as previously described[25,26]. SI_n combines four MS abundance features: peptide count, spectral count, total ion intensities and protein length. This calculation eliminates variations between replicate MS measurements and permits quantitative reproducibility and highly significant quantification of the same and different samples[25]. The equations are:

$$\mathrm{SI}=\sum_{k=1}^{\mathrm{pn}}\left(\sum_{j=1}^{\mathrm{sc}} ij\right)k$$

$$\mathrm{SI}_{\mathrm{GI}}=\mathrm{SI}/\sum_{j=1}^{n}\mathrm{SI}_j$$

$$\mathrm{SI}_n=\mathrm{SI}_{\mathrm{GI}}/L$$

where pn is the number of peptides identified for that protein, sc is the spectral count for the peptide k, i is the fragment ion intensity of peptide k, j is the jth spectral count of sc total spectral counts for peptide k, GI is the global/total intensity.

Protein mapping was performed by matching at least two peptides to a designated protein that was annotated to a gene ID. To simplify the interpretation of the complicated nanoLC-MS/MS data, pairwise comparisons were performed between any one of the meat protein groups and the casein group. Only those proteins with $P < 0.05$ were considered to be significantly different. Mascot search results are shown in the Table S2.

2.3.4 GO and KEGG pathway analysis

For interpretation of the functions of differentially expressed proteins, GO (gene ontology) and KEGG (Kyoto encyclopedia of genes and genomes) pathway analyses were performed by using the Enrichr program[27]. $P < 0.05$ in the Fisher exact test was considered for the identification of values that were significantly different.

2.3.5 Gene set enrichment analysis

Gene set enrichment analysis (GSEA) analysis was performed according to the procedure of Song et al.[28]. GSEA has multiple advantages over analysis of individual genes or proteins[29,30]. It is well accepted that the GSEA method can be applied to proteomic data, although it was initially developed for genomic data[29]. Thus, the protein set rather than the gene set was used in the present study. To interpret the GSEA results more intuitively, enrichment maps were generated with significant protein sets ($P < 0.05$, FDR < 0.25) by using the Enrichment Map plugin v2.1.026 of Cytoscape v3.2.1 (the Institute of Systems Biology, Seattle, USA).

2.4 Histological observations

The formalin-fixed jejunal tissues were embedded in paraffin and cut transversely into 7 μm thick sections by a microtome. The sections were transferred to a graded ethanol series and cleaned by xylene[11]. The sections were stained with hematoxylin and eosin. The images were captured by a light microscope (BH-2, Olympus, Tokyo, Japan) and the morphological parameters were measured with Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Villus height was measured from the baseline to the tip of the highest one. The crypt depth was measured from the baseline to the bottom of the deepest one[31]. Each tissue sample had two replicates, and ten visual fields were selected for further analysis.

2.5 Statistical analysis

The effect of diet on the variables was evaluated by one-way ANOVA and Duncan's multiple-range test with the program SAS 9.1.3 (SAS Institute Inc, Cary, NC, USA).

3 Results

3.1 PEPT1 level

The *Pept1* mRNA levels in the jejunum were higher for the beef and chicken protein groups ($P < 0.05$, Fig. 1), compared to the casein group, while no significant difference was observed for the fish and pork protein groups ($P > 0.05$, Fig. 1). Western blotting gave similar results at the PEPT1 protein level (Fig. 2). The relative PEPT1 abundance of the chicken protein group was the highest, and the lowest was observed for the pork protein group ($P < 0.05$, Fig. 2a). These results indicated that dietary protein did affect PEPT1 abundance in rat jejunum and may reflect the development of intestinal morphology.

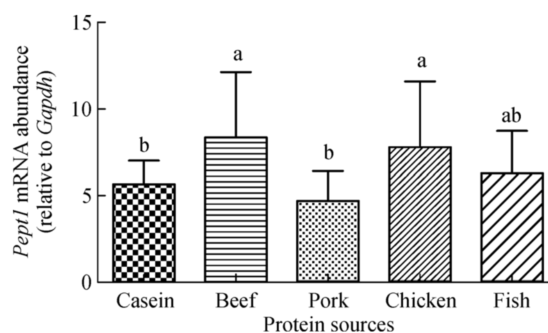

Fig. 1 PEPT1 mRNA levels in rat jejunum. *Pept1* mRNA expression was quantified by qRT-PCR. Data are shown as means with standard deviations; there were 10 biological replicates in each group; values labeled a, b, differed significantly ($P < 0.05$).

3.2 Histological parameters

Villus height differed with dietary protein ($P < 0.05$, Table 2). The chicken protein group had smaller villus height ($P < 0.05$), compared to the casein group, but no significant difference ($P > 0.05$) was observed between the beef, pork or fish protein groups and the casein group. Crypt depth did not differ ($P > 0.05$, Table 2) between any two protein groups. When data were combined, the ratio of

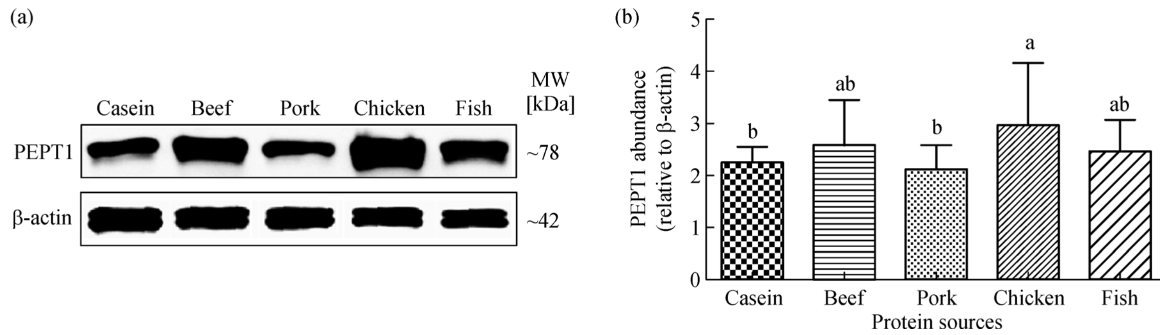

Fig. 2 PEPT1 protein levels in rat jejunum. (a) The PEPT1 band appeared at ~78 kDa and the control β-actin band appeared at ~42 kDa ; (b) the densities of PEPT1 bands were quantified and normalized to β-actin, to indicate the PEPT1 protein expression level. Data are shown as means and standard deviations, biological replicates $n = 10$ for each group; values labeled a, b, differed significantly ($P < 0.05$).

villus height to crypt depth (V/C) was the greatest for the pork protein group, but the smallest for the chicken protein group ($P < 0.05$, Table 2).

3.3 Differentially expressed proteins

Label-free LC-MS/MS identified hundreds of unique proteins from all the jejunal mucosa (Table S2). As compared to the casein group, pork protein diet had the greatest number of differentially expressed proteins, followed by chicken, fish and beef protein groups (102, 66, 53 and 49, respectively, Table S3). Hierarchical clustering revealed the differences between pork protein group and any of the other three meat protein groups

Table 2 Histological measurements of the jejunal mucosa

Dietary protein	Villus height/μm	Crypt depth/μm	V/C ratio
Casein	528 ± 53^{a}	176 ± 11	3.0 ± 0.41^{ab}
Beef protein	490 ± 66^{ab}	171 ± 11	2.9 ± 0.53^{ab}
Pork protein	545 ± 98^{a}	175 ± 6	3.1 ± 0.52^{a}
Chicken protein	450 ± 22^{b}	179 ± 18	2.5 ± 0.29^{b}
Fish protein	509 ± 42^{ab}	181 ± 25	2.9 ± 0.47^{ab}

Note: Data are presented as mean±SD, $n = 10$ for each group; values with different superscript letters indicate significant difference ($P < 0.05$).

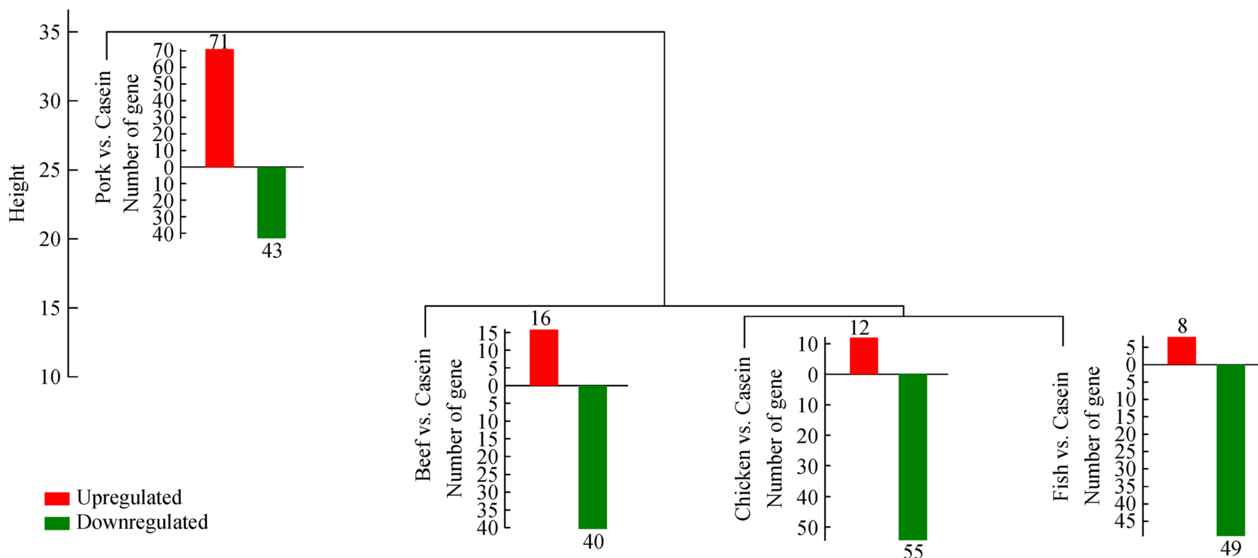

Fig. 3 Hierarchical clustering of differential membrane proteins. The four inserting coordinates show the number of upregulated (red) and downregulated (green) differential proteins. The length of vertical lines between any two coordinates indicates the differences between groups, the longer the lines were, the greater the differences were.

(Fig. 3). As compared to the casein group, the number of upregulated proteins in the pork protein group was greater than that of downregulated proteins. However, intake of beef, chicken and fish led to downregulation of a greater number of proteins.

3.4 GO and KEGG analyses

The RT-PCR, western blotting and histological results revealed significant differences in PEPT1 level and V/C ratio between chicken and pork protein groups. To obtain information about the underlying mechanism, GO and KEGG pathway analyses were performed using 66 differentially expressed proteins in the chicken protein group and 102 proteins in the pork protein group. As compared to the casein group, the chicken protein group showed significant differences in biological process, cellular component and molecular function (Tables 3–5). However, no significant differences were observed between the pork protein group and the casein group (data not shown).

In biological process categories, the enriched GO terms derived from upregulated proteins were related to cell junction organization, Fc-gamma receptor signaling pathway regulation of proton transport and oxidative phosphorylation ($P < 0.05$, Table 3). The terms derived from downregulated proteins were relevant to metabolic processes related to organic acids (e.g., uronic acid), protein polymerization and folding, digestion, and terpenoid and GTP metabolic process ($P < 0.05$, Table 3).

In cellular component categories, the terms derived from upregulated proteins were involved in the mitochondrial respiratory chain and cytochrome complex ($P < 0.05$, Table 4). The terms derived from downregulated proteins were associated with myosin complex, brush border, NADH dehydrogenase and respiratory chain complex ($P < 0.05$, Table 4).

In molecular function categories, the terms derived from upregulated proteins were related to oxidoreductase activity, ubiquinol cytochrome-c reductase activity and amide transmembrane transporter activity ($P < 0.05$, Table 5). The downregulated proteins were associated with organic acid binding, drug binding, electron carrier activity, fatty acid transporter activity, calcium-transporting ATPase and dipeptidyl peptidase activity ($P < 0.05$, Table 5).

Table 3 Enriched GO terms in biological process categories for differential proteins

Biological process _ GO term (GO ID)	Overlap	*P*-value
Chicken protein vs. Casein _ upregulated		
Cell junction organization (GO:0034330)	2/189	0.011
Immune response-regulating cell surface receptor signaling pathway involved in phagocytosis (GO:0002433)	2/203	0.012
Fc-gamma receptor signaling pathway involved in phagocytosis (GO:0038096)	2/203	0.012
Fc-gamma receptor signaling pathway (GO:0038094)	2/203	0.012
Fc receptor mediated stimulatory signaling pathway (GO:0002431)	2/204	0.012
Regulation of proton transport (GO:0010155)	1/14	0.013
Oxidative phosphorylation (GO:0006119)	1/14	0.013
Chicken protein vs. Casein _ downregulated		
Cellular glucuronidation (GO:0052695)	4/17	< 0.0001
Uronic acid metabolic process (GO:0006063)	4/18	< 0.0001
Glucuronate metabolic process (GO:0019585)	4/18	< 0.0001
Flavonoid metabolic process (GO:0009812)	3/11	< 0.0001
Monocarboxylic acid metabolic process (GO:0032787)	9/473	< 0.0001
Metinoic acid metabolic process (GO:0042573)	3/21	< 0.0001
Protein polymerization (GO:0051258)	4/64	< 0.0001
Cellular protein complex assembly (GO:0043623)	6/256	0.0003
'*De novo*' posttranslational protein folding (GO:0051084)	3/49	0.0007
'*De novo*' protein folding (GO:0006458)	3/54	0.001
Diterpenoid metabolic process (GO:0016101)	4/87	0.0003
Terpenoid metabolic process (GO:0006721)	4/97	0.0004
GTP metabolic process (GO:0046039)	5/258	0.002
GTP catabolic process (GO:0006184)	5/243	0.002
Digestion (GO:0007586)	3/66	0.002

Note: Overlap represents the numbers of identified proteins matching with GO terms.

Table 4 Enriched GO terms in cellular component categories for differential proteins

Cellular component_ GO term (GO ID)	Overlap	*P*-value
Chicken protein vs. Casein _ upregulated		
Mitochondrial respiratory chain complex III (GO:0005750)	1/8	0.008
Respiratory chain complex III (GO:0045275)	1/8	0.008
Mitochondrial respiratory chain (GO:0005746)	1/14	0.014
Cytochrome complex (GO:0070069)	1/18	0.017
Chicken protein vs. Casein _ downregulated		
Myosin complex (GO:0016459)	4/64	< 0.0001
Myosin II complex (GO:0016460)	3/25	0.0001
Muscle myosin complex (GO:0005859)	2/18	0.002
Mitochondrial nucleoid (GO:0042645)	3/40	0.0005
Brush border (GO:0005903)	2/24	0.003
NADH dehydrogenase complex (GO:0030964)	2/47	0.013
Respiratory chain complex I (GO:0045271)	2/47	0.0123

Note: Overlap represents the numbers of identified proteins matching with GO terms.

Table 5 Enriched GO terms in molecular function categories for differential proteins

Molecular function_ GO term (GO ID)	Overlap	*P*-value
Chicken protein vs. Casein _ upregulated		
Oxidoreductase activity, acting on diphenols and related substances as donors, cytochrome as acceptor (GO:0016681)	1/8	0.008
Oxidoreductase activity, acting on diphenols and related substances as donors (GO:0016679)	1/9	0.009
Ubiquinol-cytochrome-c reductase activity (GO:0008121)	1/8	0.008
Amide transmembrane transporter activity (GO:0042887)	1/10	0.010
Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor (GO:0016620)	1/31	0.030
Oxidoreductase activity, acting on the aldehyde or oxo group of donors (GO:0016903)	1/42	0.039
Chicken protein vs. Casein _ downregulated		
Monocarboxylic acid binding (GO:0033293)	6/59	< 0.0001
Isoprenoid binding (GO:0019840)	5/38	< 0.0001
Retinoid binding (GO:0005501)	5/38	< 0.0001
Retinoic acid binding (GO:0001972)	4/23	< 0.0001
Carboxylic acid binding (GO:0031406)	6/200	0.0001
Organic acid binding (GO:0043177)	6/201	0.0001
Fatty acid binding (GO:0005504)	3/26	0.0002
Drug binding (GO:0008144)	4/93	0.0005
Voltage-gated anion channel activity (GO:0008308)	3/21	0.0001
Wide pore channel activity (GO:0022829)	3/23	0.0001
Anion channel activity (GO:0005253)	3/80	0.004
Voltage-gated ion channel activity (GO:0005244)	3/189	0.037
Voltage-gated channel activity (GO:0022832)	3/189	0.037
Electron carrier activity (GO:0009055)	4/107	0.0008
Calcium-transporting ATPase activity (GO:0005388)	1/9	0.038
Fatty acid transporter activity (GO:0015245)	1/9	0.038
Dipeptidyl-peptidase activity (GO:0008239)	1/11	0.045

Note: Overlap represents the numbers of identified proteins matching with GO terms.

The enriched KEGG pathways derived from upregulated proteins were related to adherens junctions, focal adhesion, hippo signaling pathway, apoptosis, collecting duct acid secretion and galactose metabolism ($P < 0.05$, Table 6). The downregulated proteins were related to glycolysis, retinol metabolism, ascorbate and aldarate metabolism, xenobiotics and drug metabolism.

Table 6 Enriched KEGG pathway for differential proteins

KEGG Term	Overlap	*P*-value
Chicken protein vs. Casein _ upregulated		
Adherens junctions	2/74	0.004
Focal adhesion	2/202	0.026
Hippo signaling pathway	2/153	0.016
Apoptosis	2/140	0.013
Collecting duct acid secretion	1/27	0.035
Galactose metabolism	1/30	0.039
Chicken protein vs. Casein _ downregulated		
Starch and sucrose metabolism	6/56	2.24×10^{-6}
Pentose and glucuronate interconversions	5/36	5.30×10^{-6}
Fructose and mannose metabolism	2/32	0.02
Ascorbate and aldarate metabolism	5/27	1.49×10^{-6}
Retinol metabolism	5/65	7.47×10^{-5}
Drug metabolism- other enzymes	5/46	1.59×10^{-5}
Drug metabolism- cytochrome P450	5/69	9.76×10^{-5}
Metabolism of xenobiotics by cytochrome P450	5/73	0.0001

Note: Overlap represents the numbers of identified proteins matching with KEGG terms.

3.5 GSEA analysis

GSEA analysis was performed in order to analyze biological functions at the level of protein sets instead of individual proteins. There were 4, 8, 10 and 5 protein sets, respectively in the beef, pork, chicken and fish protein groups that differed from those in the casein group (Table S4). The clustering and enrichment maps revealed that intake of four meat proteins regulated carbohydrate metabolism, biological oxidation, biological development and several signaling pathways (Figs. 4–5).

When compared to the casein group, the cluster of protein sets related to carbohydrate metabolism was significantly changed by intake of pork protein, which is reflected by increased glucose metabolism, glycogen storage, and starch and sucrose metabolism. Intake of chicken increased glucose metabolism, but inhibited starch and sucrose metabolism. Ingestion of fish protein inhibited pentose and glucoronate interconversions, and ascorbate and aldarate metabolism. Beef protein showed a high similarity to casein.

For the protein sets related to biological oxidation, as compared to control (casein), intake of chicken protein increased NFE2L2, drug metabolism and metabolism of xenobiotics by cytochrome P450, but decreased oxidative phosphorylation. However, intake of pork protein inhibited respiratory electron transport. Intake of beef protein also inhibited respiratory electron transport, but increased NFE2L2. Fish protein intake did not affect any protein sets related to biological oxidation.

In addition, intake of chicken protein inhibited the protein sets related to gap junction, developmental biology and axon guidance. Intake of fish protein did not inhibit the protein sets related to gap junction, but inhibited cGMP-PKG signaling pathway and GPCR downstream signaling pathway.

4 Discussion

The jejunum is the main site for nutrient absorption/transport and its development affects bioavailability of food nutrients[32]. Villus height and crypt depth may reflect the development of the small intestine. Greater V/C ratio indicates higher capacity of nutrient absorption and transport from the intestinal lumen to the bloodstream[10,11]. During maturation, the expression of proteins related to brush border assembly, metabolism of glucose, amino acids, and fatty acids increases in intestinal epithelial cells along the crypt-villus axis[33,34]. PEPT1 is located in the brush border membrane of enterocytes and is responsible for the transport of dipeptides and tripeptides[8,9]. A 7-day feeding study found lower V/C ratio but higher abundance of PEPT1 in the jejunum for the rats fed beef protein than those of the rats fed pork, chicken and fish proteins[20]. In the present study, the chicken protein group had higher levels of PEPT1 mRNA and protein, but lower V/C ratio than the pork protein group. Compared to casein, intake of chicken protein had a stronger influence on PEPT1 level and morphology of rat jejunum than in the pork protein group. This could be because the products of protein digestion are likely to be the metabolic signals triggering the modulation of PEPT1 expression[35], and proliferation and differentiation of the intestinal epithelial cells[36]. In addition, the gastric leptin may selectively mediate peptide transport across the intestinal epithelium[37].

Diet has a broad influence on the small intestine. For example, protein limitation in diet can upregulate the proteins involved in protein/carbohydrate digestion, intestinal mucosal tight junction and cell adhesion, and the immune response to foreign antigens in the jejunal mucosa of pigs, but downregulate amino acid transport, innate and auto immunity, as well as cell proliferation and apoptosis[38]. In the present study, GO and KEGG pathway analyses indicated that, under the normal dose, intake of chicken protein induced upregulation of respiratory chain, oxidative phosphorylation and oxidoreductase activity, cell

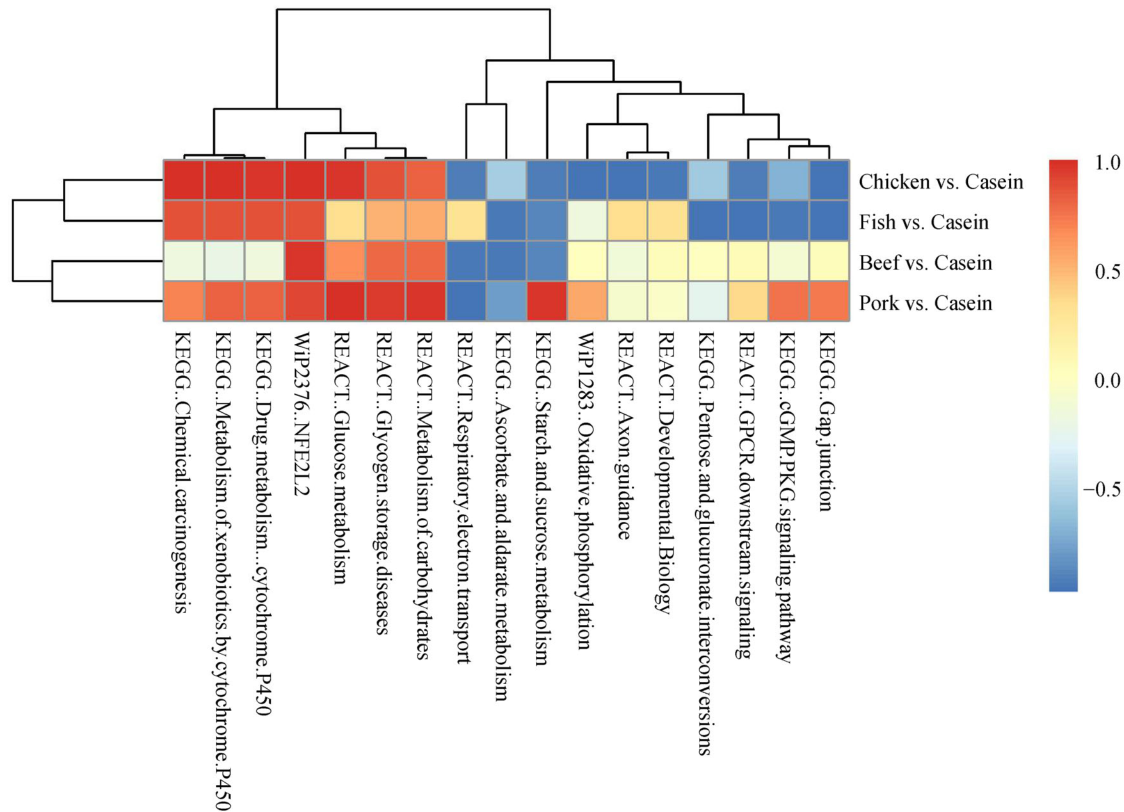

Fig. 4 Clustered heat map of jejunal membrane protein sets. The rows are the clusters of differential protein sets (pathways) responding to one meat protein diet as compared to the casein group, and the columns are individual protein sets (pathways). The color scale represents the fraction of regulated protein sets; the more intense the color, the higher the fraction. Red means enriched, while blue means suppressed as compared to casein.

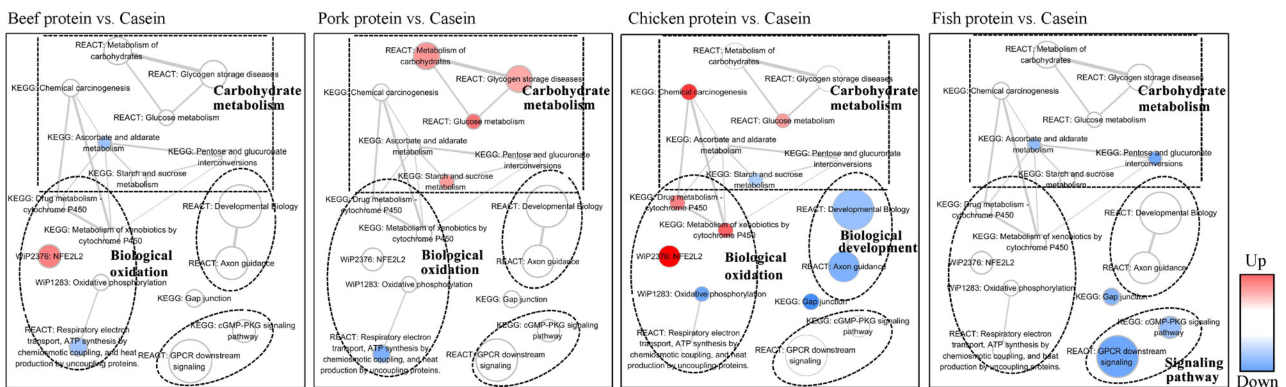

Fig. 5 Network of protein sets for different protein groups enriched by GSEA analysis. The network was produced using Cytoscape. Nodes represent the enriched protein sets. The red nodes represent upregulated protein sets and the blue nodes represent downregulated protein sets. The sizes of nodes are proportional to the total number of proteins within each set (from 15 to 500). The lines between nodes represent the overlap score (Jaccard and overlap coefficient > 0.375) depending on the number of proteins that two protein sets share.

junction organization, and regulation of proton transport. This is closely linked to the process of biologic oxidization[39], accompanied by energy release to meet immediate energy needs of all the biological reactions[40]. This process would further regulate amide transmembrane transporter activity, glucose and oligopeptide transporter expression, galactose metabolism, and consequently the development of intestinal morphology. Glucose-mediated

energy homeostasis is an upstream event involved in regulation of the Hippo signaling pathway[41], which can regulate cell proliferation and apoptosis of organs. Consequently, intake of different meat proteins could change jejunal absorption capacity by regulating intestinal morphology.

In addition, dietary proteins also regulated intercellular substance transport and signal transduction (cell junction organization, adherens junctions, and focal adhesion) and then collecting duct acid secretion via neural control. This can be attributed to the differences in amino acid composition of different meats. For example, L-glutamine can regulate the expression of intestinal tight junction protein in weanling piglets[42]. Dietary N reduction led to the downregulation of cadherin 17[43]. Intake of chicken protein can increase focal adhesion, prevent insulin resistance, and consequently increase glucose metabolism[44].

Also, intake of chicken protein reduced carbohydrate metabolism, ascorbate and metabolism of aldarate, retinol, and xenobiotics. These processes can be realized by inhibiting changes in regulating respiratory chain complexes, anion channels, and transporter activity. Peptides derived from dietary chicken protein might reduce changes in regulating calcium-transporting ATPase activity to inhibit calcium transport, dipeptidyl peptidase activity to promote dipeptide transport, and fatty acid transporter activity to inhibit fatty acid transport.

5 Conclusions

The types of dietary proteins had a significant effect on PEPT1 expression and proteome profile of rat jejunum. When compared to casein, intake of chicken protein had the strongest effect on PEPT1 expression level and V/C ratio of the jejunum, but the weakest for the pork protein group. Intake of chicken protein increased oligopeptide transport by increasing PEPT1 expression level, downregulating biological processes involving energy production and reducing dipeptidyl peptidase activity. Also, it decreased oligopeptide absorption capacity by inducing the Hippo signaling pathway related to intestinal morphology. These findings provide new insights into protein nutrition.

Supplementary materials The online version of this article at https://doi.org/10.15302/J-FASE-2018206 contains supplementary materials (Tables S1–S4).

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Compliance with ethics guidelines Mengjie Li, Chunbao Li, Shangxin Song, Xinglian Xu, and Guanghong Zhou declare that they have no conflicts of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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