Effect of glucose, soy oil, and glutamine on protein expression and mTORC1 pathway of jejunal crypt enterocytes in weaned piglets[1]

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ABSTRACT

This study was conducted to evaluate the effects of glucose, soy oil, or glutamine on jejunal morphology, protein metabolism, and protein expression of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway in jejunal villus or crypt compartment of piglets. Forty-two 21 d-weaned piglets were randomly allotted to one of the three isocaloric diets formulated with glucose, soy oil, or glutamine for 28 days. On day 14 or 28, the proteins in crypt enterocytes were analyzed with isobaric tags for relative and absolute quantification, and proteins involved in mTORC1 signaling pathway in villus or crypt compartment cells were determined by western blotting. Our results showed no significant differences ($P > 0.05$) in jejunal morphology among the three treatments on day 14 or 28. The differentially expressed proteins mainly took part in a few network pathways, including antimicrobial or inflammatory response, cell death and survival, digestive system development and function, and carbohydrate metabolism. On day 14 or 28, there were higher protein expression of 4EBP1 in jejunal crypt compartment of piglets supplemented with glucose or glutamine compared with soy oil. On day 28, higher protein expression of p-mTOR in crypt compartment was observed in piglets supplemented with glucose compared with the soy oil. In conclusion, the isocaloric glucose, soy oil, or glutamine did not affect the jejunal morphology of piglets; however, they had different effects on the protein metabolism in crypt compartment. Compared to soy oil, glucose or glutamine may be better energy supplies for enterocytes in jejunal crypt compartment.

Key words: glucose, glutamine, intestine, mTOR, soy oil, weaned piglets

Early weaning (14- to 21-day weaning age) is one of the most stressful events that pigs encounter in swine production, because weaned pigs must rapidly adapt to the changes in diet, physical environment, and social environment [1, 2]. Weaned piglets have reduced feed intake immediately after weaning, which is highly associated with the negative changes in intestinal architecture and function, such as villous atrophy and impairment of intestinal barrier function, decreased enzymatic activities, and increased expression of proinflammatory cytokines [3, 4]. The metabolism shifts in the intestinal epithelium play a significant role in maturation of the intestinal epithelium [5, 6]. Our previous study has also reported that weaning influences energy and protein metabolism in the intestinal epithelium, which further impacts the proliferation of intestinal epithelial cells in weaned piglets [4].

Intestinal renewal is critically important in intestinal development, maintenance, and recovery from tissue damage, therefore, the intestinal epithelial cells have notably high energy demands due to the rapid renewal [1, 7]. Glutamate, aspartate, and glutamine are the major oxidative substrates utilized by the intestinal epithelial cells, whereas glucose and fatty acids are minor oxidative substrates [8, 9]. However, our previous study has reported that weaned piglets supplemented with glucose, soy oil, or glutamine exhibited different responses. Pigs fed with glucose or soy oil had better growth performance due to enhanced feed intake, whereas pigs supplemented with glutamine have improved immunity [10]. Glucose, fatty acids, and glutamine have different metabolism pathways, thus they may have different physiological functions [11-13]. On the basis of previously published research, we hypothesized that the intestinal metabolism may be altered at the organismal level in those pigs. The mammalian target of rapamycin (mTOR) is a crucial signaling node that integrates

environmental cues to regulate cell survival, proliferation, and metabolism. Lysosomal mTOR complex1 (mTORC1) modulates the cell response to growth factors and nutrients by increasing protein synthesis and cell growth, and repressing the autophagy-lysosomal pathway [14]. Thus, the present study aims to investigate the effects of dietary supplementation of glucose, soy oil, or glutamine on protein expression in crypt compartment and the mTORC1 signaling pathway along the jejunal crypt–villus axis in weaned piglets.

Methods

Animals and experimental treatments

The experimental design and procedures used in this study were approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. Weaned piglets used in this experiment were bred from Duroc boars to Landrace × Large York shire sows. A total of 42 piglets were weaned at 21 d of age, grouped by body weight and sex and were randomly assigned to one of three treatments. Each treatment group had 14 piglets, including seven gilts and seven barrows. They were fed isocaloric diets supplemented with either glucose, soy oil, or glutamine. Glucose, soy oil, and glutamine accounted 2.5% of metabolic energy (ME) in each diet. Glucose and soy oil were edible grade and refined, and glutamine was obtained from Sigma (G3126). The diets were formulated to meet the nutrient requirements for weaned piglets (NRC, 2012). The nutrient composition is depicted in Supplemental Table. The experiment was conducted for 28 days with two phases, and two weeks in each phase. There were no differences in initial body weight among glucose, soy oil, and glutamine treatments (7.36 ± 0.23 vs. 7.39 ± 0.19 vs. 7.43 ± 0.17; p = 0.968), and there were significant differences in body weight on day 14 (9.84 ±

0.30 vs. 10.03 ± 0.30 vs. 9.08 ± 0.22, $p = 0.046$) but not on day 28 (14.77 ± 0.53 vs. 14.73 ± 0.62 vs. 13.50 ± 0.49, $p = 0.208$).

Animal management and sample collection

The piglets were housed individually in an environmentally controlled nursery room with hard plastic slatted flooring. All piglets had free access to drinking water. On day 14 or 28, seven piglets (four barrows and three gilts) were randomly selected from each treatment and were euthanized for tissue sampling. The remained pigs were euthanized after the completion of the experiment (day 28). Intestinal segments were collected from the mid-jejunum, rinsed with ice-cold phosphate buffered saline, and divided into two sections. One (approximately 1–2 cm) section was fixed with 4% formaldehyde-phosphate buffer and kept at 4°C for microscopic assessment of the mucosal morphology. The other section was used for sequential isolation of small intestinal epithelial cells.

Sequential isolation of small intestinal epithelial cells along the crypt–villus axis was performed according to our published research [15]. In brief, the jejunum was flushed once with phosphate-buffered saline and then incubated at 37°C for 30 min in 15 mL of oxygenated phosphate-buffered saline. After the pre-incubation, the jejunal segments were then filled with 15–30 mL of isolation buffer [5 mM Na_2EDTA, 10 mM HEPES, 0.5 mM DTT, 0.25% BSA, 2.5 mM D-glucose, 2.5 mM L-glutamine, 0.5 mM DL-β-hydroxybutyrate sodium salt, oxygenated with an O_2/CO_2 mixture (19:1 vol/vol)] for sequential isolation of three epithelial cell fractions (upper villus: F1; middle villus: F2; crypt: F3) from the villus tip to the crypt bottom. Isolated cell fractions were pooled for each piglet. After that, the cell fraction was washed twice with an oxygenated cell suspension buffer (155 mM KCl at pH

7.4), and centrifuged at 400 g for 4 min at 4°C. The washed cells were immediately frozen by liquid nitrogen and stored at -80°C for further use.

Measurement of jejunal morphology

The cross-sections of intestinal samples preserved in 4% formaldehyde-phosphate buffer were prepared using standard paraffin embedding techniques. The samples were sectioned at 5 μm thickness and were stained with hematoxylin and eosin (HE) [16]. The villus height and crypt depth were measured in the stained sections under a microscope with 40× combined magnification using an image processing and analysis system (version 1; Leica Imaging Systems Ltd., Cambridge, UK). At least fifteen well-oriented intact villi and the associated crypt depths were measured in each section for each piglet.

Sample preparation and isobaric labeling

Briefly, the crypt cells were resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% wt/vol 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate, 20 mM tributyl phosphate, and 0.2% Bio-lyte (pH 3–10)) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The cells were disrupted with disposable tissue-grinding pestles for 5 min and then treated by ultra sonication (Sonics & Material, Newtown, CT, USA) for 10 min. Trypsin digestion and iTRAQ labeling were performed according to the manufacturer's protocol (Applied Biosystems Inc., Foster City, CA, USA) and dried under vacuum.

High pH reverse phase separation and low pH nano-HPLC-MS/MS analysis

The iTRAQ labeled samples were divided into 12 fractions by high pH separation using a Aquity ultra performance liquid chromatography system (Waters Corporation,

Milford, MA) that was connected to a reverse phase column (BEH C18 column, 2.1mm × 150 mm, 1.7-μm) (Waters Corporation, Milford, MA) and dried under vacuum. High pH separation was performed using a linear gradient (0% ~45%) for 45 min in water-ammonium formate-acetonitrile solvents (pH 10.0).

Each fraction was dissolved in solvent A (5% acetonitrile, 0.1% formic acid) and analyzed on TripleTOF 5600 systems (AB SCIEX) in an information dependent mode. Briefly, peptides were separated on reverse-phase column (ZORBAX 300SB-C18 column, 5μm, 0.1 × 15 mm; Micromass) using an Eksigent 1D PLUS system (AB SCIEX) at an analytical flow rate of 300 nL/min. The peptides were separated with 120 min linear gradient from 5% to 40% solvent B (0.1% formic acid/90% acetonitrile). Survey scans were acquired from 400 to 1500 with up to 15 precursors selected for MS/MS and dynamic exclusion for 20 sec.

Database Analysis and Quantification

Mascot software (version 2.3.02, Matrix Science) was used to simultaneously identify proteins. The search parameters were as follows: 1) Uniprot pig database; 2) peptide tolerances was set as 25 ppm and MS/MS tolerance was set as 0.2 Da; 3) the fixed modifications of MMTS were set as cysteine; and oxidation of methionine was considered as a variable modification. Scaffold was used to quantify the isobaric tag peptide and for protein identification. For protein identification, the filters were set as follows: significance threshold $P < 0.05$ (with 95% confidence) and ion score or expected cutoff < 0.05 (with 95% confidence). For protein quantitation, the filters were set as follows: “median” was chosen for the protein ratio type; the minimum precursor charge was set to 2 and minimum peptides

were set to 2; only 2 and ＞2 unique peptides were used to quantify proteins. The median intensities were set as normalization, and outliers were removed automatically. The peptide threshold was set as above for identity. Quantitative ratios were $\log_2$ normalized for final quantitative testing. The normality of data was checked with Shapiro–Wilk test and then analyzed using *t*-test, Mann–Whitney test, and ANOVA (followed by a Tukey–Kramer post-hoc test).

Bioinformatics Analysis

The differential proteins were blasted against Swiss-Prot human database. The Ingenuity Pathway Analysis (content version: 47547484) was used to classify these identified proteins and enrich networks.

Expression of mTORC1 signaling pathway related proteins in weaned piglets

The mammalian target of rapamycin signaling pathway (mTOR), phosphor-mTOR, eukaryotic initiation factor-4E binding protein-1 (4EBP1), phosphor-4EBP1, S6 kinase (S6K), phosphor-S6K, AMP-activated protein kinase (AMPK) and β-actin protein abundances in enterocytes along the crypt–villus axis of the jejunum were determined by western blotting as previously reported [(15)]. Total proteins were extracted using ice-cold radioimmunoprecipitation assay lysis buffer (Biyuntian, Shanghai) containing phenylmethylsulfonyl fluoride. The sample was then centrifuged, and the resulting supernatants were sampled for analyses of their protein concentrations before Western blotting of the target proteins.

The following antibodies were used for western blot analysis. Antibodies for mTOR, phospho-mTOR (Ser2448), 4EBP1 , phospho-4EBP1 (Thr70), S6K, phospho-S6K

(Thr421/Ser424, Thr389) and AMPK, and β-actin were purchased from Cell Signaling Technology (Cedarlane, ON, Canada). β-actin was used to normalize the abundance of the target proteins. Quantification of bands was carried out using Image J software (Bio-Rad).

Statistical analysis

The intestinal morphology and western blot data were analyzed using two-way ANOVA in SAS software (Version 9.2; SAS Inst. Inc., Cary, NC). Values are mean ± SEM. *P*-values < 0.05 were used to indicate statistical significance.

Results

Jejunal morphology

There were no significant differences ($P > 0.05$) in villus height, crypt depth and villus height:crypt depth (V:C) of the jejunum among the three treatments on days 14 and 28 (Table 1; Supplemental Figure 1).

Differentially expressed proteins in the crypt compartment cells in the glucose, soy oil, and glutamine groups

A total of 5,200 proteins were identified in the present study using Mascot version 2.3.2. A protein with ≥ 1.2-fold or ≤ 0.8-fold difference and a *P* value ≤ 0.05 was regarded as being differentially expressed. A total of 137 differentially expressed proteins were identified in the crypt compartment of pigs among the three groups on day 14. The highest number of differentially expressed proteins were between the glucose and glutamine groups on day 14. These 137 differential proteins were classified into 23 groups based on major intestinal functions, i.e., transcription; metabolism of protein, amyloidosis, processing of RNA, catabolism of protein, degranulation of cells, fatty acid metabolism, repair of DNA,

deubiquitination of protein, synthesis of protein, processing of rRNA, stabilization of protein, cell spreading, cytolysis, nonsense-mediated mRNA decay, organization of actin cytoskeleton, replication of viral replicon, replication of flaviviridae, stabilization of mRNA, metabolism of triacylglycerol, oxidation of fatty acid, or reorganization of cytoskeleton and synthesis of amine (Figure 1.a).

A total of 56 differentially expressed proteins were identified in the crypt compartment of pigs from the three groups on day 28. The highest number of differential proteins were between the soy oil and glutamine groups. These 56 differential proteins were classified into 23 groups based on major intestinal function, i.e., transcription of DNA, G1 phase, metabolism of membrane lipid derivative, synthesis of carbohydrate, concentration of lipid, deubiquitination of protein, metabolism of DNA, proliferation of lymphocytes, primary adenocarcinoma, apoptosis of thyroid tumor cell lines, cell viability of monocyte-derived dendritic cells, chemotaxis of monocytes, chronic myelomonocytic leukemia, concentration of triacylglycerol, congenital anemia, differentiation of stem cell lines, differentiation of keratinocytes, fragmentation of DNA, G1 phase of pancreatic cancer cell lines, hydrolysis of triacylglycerol, or infection of hepatoma cell line and invasion of colorectal cancer cell lines (Figure 1.b).

The differential proteins between the soy oil and glutamine groups on day 14 or 28 were also networked on Table 2. These differential proteins mainly took part in few networks, including antimicrobial response, inflammatory response, cell death and survival, digestive system development and function, carbohydrate metabolism.

mTORC1 pathway in villus and crypt compartment by western blotting

On day 14, the protein expression of mTOR and p-mTOR was higher ($P < 0.05$) in the jejunal villus compartment of pigs supplemented with glucose than pigs in the glutamine group, whereas pigs in the soy oil group had the lowest expression (Figure 2.a). 4EBP1 and AMPK expression was higher ($P < 0.05$) in the jejunal crypt compartment of pigs in the glucose and glutamine groups than pigs in the soy oil group (Figure 2.c).

On day 28, pigs fed with glucose had greater ($P < 0.05$) protein expression of mTOR than pigs fed with soy oil, and had greater ($P < 0.05$) AMPK expression than pigs fed with glutamine (Figure 2.b). Pigs fed with glutamine had greater ($P < 0.05$) protein expression of 4EBP1 in the jejunal crypt compartment than pigs in the soy oil group. The protein expression of p-mTOR was the highest ($P < 0.05$) in the jejunal crypt compartment of pigs fed glucose than pigs fed with soy oil or glutamine (Figure 2.d).

Discussion

Weaning stress is characterized by poor growth, decreased feed intake, and diarrhea [17]. The intestine has a high rate of energy expenditure for performing its digestion and absorption processes. Inadequate food intake in weaned piglets may contribute to intestinal dysfunction and morphology atrophy [18]. Three types of substrates (amino acids, glucose, and fatty acids) are oxidized by intestinal tissues as the energy source, in which amino acids (i.e., glutamine, glutamate, and aspartate) are the major contributors to mucosal oxidative energy generation [8, 9]. Our previous study reported that weaned piglets supplemented with glucose or soy oil demonstrated better growth performance possibly due to enhanced feed intake, whereas those supplemented with glutamine may have improved immunity and intestinal oxidative capacity [10]. In the present study, we aimed to investigate the effects of these three

energy sources on intestinal function, which may attribute to the changes of performance and intestinal health in weaned pigs.

The intestinal villus height to crypt depth ratio is widely adopted to assess the nutrient digestion and absorption capacity of the small intestine [19]. Given the better growth rate in pigs fed with glucose and soy oil on day 14, it is reasonable to expect that the intestinal morphology of pigs may be altered. However, there were no significant differences in the intestinal morphology among the three groups on day 14. The reason may be that glutamine, the main respiratory substrate of enterocytes, has a better energy supply capacity in enterocytes than soy oil or glucose and it promoted the recovery of intestinal morphology. Intestinal epithelial cells in the crypt proliferate in weaned piglets in response to weaning [4]. Enteral nutrients play an important role in regulating intestinal protein accretion, epithelial cell proliferation, and mucosal growth [3]. In the present study, we isolated and identified the proteins being expressed in jejunal crypt epithelial cells of the pigs supplemented with glucose, soy oil, or glutamine. There were more differential expressed proteins in the crypt compartment of the three groups on day 14 than on day 28. There were more proteins involved in the nutrient metabolism or repair of DNA on day 14 expressed in the three groups. However, the main function of the differentially expressed proteins on day 28 included transcription or metabolism of DNA. These results indicated that the protein metabolism of enterocytes in the crypt compartment was adjusted after a 28-day period among the three groups. In addition, the network analysis of the differentially expressed proteins showed that there were differences in the antimicrobial response between soybean oil or glucose versus glutamine. Paneth cells are specialized epithelial cells located at the base of small intestinal

crypts that act as important effectors of innate immunity through their secretion of antimicrobial peptides [20]. Glutamine has been found to increase antimicrobial peptide expression and reduce gut inflammation in animal models of inflammatory bowel diseases [21]. Thus we speculated that Gln may affect the expression of antimicrobial peptides through the Paneth cells in the crypt compartment of weaned piglets.

mTOR, as the catalytic subunit of two distinct protein complexes, mTORC1 and mTORC2, is the major regulator of growth in animals and is the key link between the availability of nutrients in the environment and the control of most anabolic and catabolic processes [22, 23]. Nutrient signals carried by free amino acids and glucose regulate mTORC1 activation through the Rag guanosine triphosphatases [24]. Given the central role of mTORC1 in promoting lipid biosynthesis, dietary lipids may provide feedback on mTORC1 activation. However, whether and how mTORC1 senses dietary lipids in a cell-autonomous manner remains unclear [25]. The 4EBP1 and the translation regulator S6K are two key downstream targets of mTOR [26]. Our previous study showed that the mTOR signaling pathway may be involved in regulating intestinal epithelial cell renewal along the crypt–villus axis [4]. The present study also showed that glucose, soy oil, and glutamine had differential effects on the related protein expression of the mTORC1 pathway in the jejunum of weaned piglets. On day 14 or 28, piglets supplemented with glucose or glutamine had higher protein expression of 4EBP1 in the jejunal crypt compartment compared with the soy oil group. On day 28, piglets supplemented with glucose had higher protein expression of p-mTOR in the jejunal crypt compartment compared with the soy oil group. These results may indicate that dietary glucose or glutamine activated mTORC1 and increased the related protein expression of

mTORC1 signaling pathway. Compared with suckling piglets, proteins involved in the tricarboxylic acid and β-oxidation were significantly downregulated, but glycolysis was significantly upregulated in crypt cells of the early-weaned piglets [15]. Villus cells use luminal nutrients as well as arterial nutrients, while crypt cells use arterial nutrition [27]. The suckling-weaning transition coincides with metabolic changes that are probably related to the metabolism difference between villus and crypt cells. Our present results also indicated that enterocytes in crypt compartment may make better use of glucose or glutamine than soy oil.

In conclusion, these results indicated that isocaloric glucose, soy oil, or glutamine had no effect on jejunal morphology of weaned piglets; however, they had different effects on protein metabolism in crypt compartment. Compared with soy oil, glucose or glutamine may be better energy supplies for enterocytes in jejunal crypt compartment. Pig is a good model for human nutrition and medicine, and the pig gastrointestinal tract is much closer to that in humans than that in rodents. Thus the present finding may be useful for human in stress to develop future therapeutic interventions.

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The author's contributions were as follows: X. X. and Y.-L. Y. designed the study; X. X., D.-H. L., and D.-F. X. performed the experiments and collected samples; X. X. and D.-H. L.,

analyzed the data and wrote the manuscript; and X. X., L.-J. Z., M.-H. S., Y.-H. L. and Y.-L. Y. revised the manuscript.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1. Effects of dietary supplementation with different energy sources on jejunal morphology of weaned piglets[1]

Item[2]	day 14			day 28			SEM	*P*-value		
	Glucose	Soy oil	Glutamine	Glucose	Soy oil	Glutamine		treatment	day	treatment x day
VH, μm	317.96	283.64	279.00	332.55	305.08	342.82	11.43	0.356	0.053	0.359
CD, μm	168.29	180.66	182.82	210.03	182.41	190.05	6.27	0.688	0.056	0.216
VH:CD	2.08	1.67	1.65	1.75	1.75	1.95	0.10	0.593	0.950	0.165

[1]Seven piglets per treatment.

[2]VH: villus height, CD: crypt depth

Table 2. Differentially expressed proteins involved in each network of jejunal crypt cells among the three groups [1]

functional network		molecules in network[2]
Soybean oil vs glutamine		
day 14	Antimicrobial Response; Inflammatory Response	*BANF1;DHX58;FABP2;GMPR;IFI44L;IFIT1;IFIT3;SERPINC1;USP 18;VIM*
day 28	Cell Death and Survival; DNA Replication; Recombination, and Repair	*ARAP1;BAK1;CBLB;COMMD7;HMGA1;LPIN2;MVP*
Glutamine vs glucose		
day 14	Inflammatory Response; Antimicrobial Response	*APOH;GZMA;HIST1H1B;IFIT3;OASL;PDE6D;TMSB10*
	Cell Death and Survival; Organismal Injury	*BRCC3;DIMT1;EPC1;GPX2;PARP;TBL1XR1;XAF1;ZBTB7A*
	Digestive System Development and Function;	*CKAP4;DEGS1;GNLY;GPD2;TSPO*
	Cellular Function and Maintenance; Cellular Movement	*COMMD6;FABP2;PARP12;SLC35G1;USP18*
day 28	Cell Death and Survival; Carbohydrate Metabolism	*AFAP1L2; ARAP1;H2AFY; HMGA1*
Soybean oil vs glucose		
day 14	Immunological Disease; Infectious Diseases	*ALOX15B;CCNH;CD3E;COMMD6;EXOSC7;MORF4L1;MX1*
	Cell-To-Cell Signaling and Interaction; Immune Cell Trafficking	*POLD3;SERPINC1;UHRF1;ZYX*
day 28	Organismal Survival; Cell Death and Survival; Antimicrobial Response	*BAK1;H2AFX;MX2;OASL*

[1]Seven piglets per treatment.

[2]Gene name

Figure legend

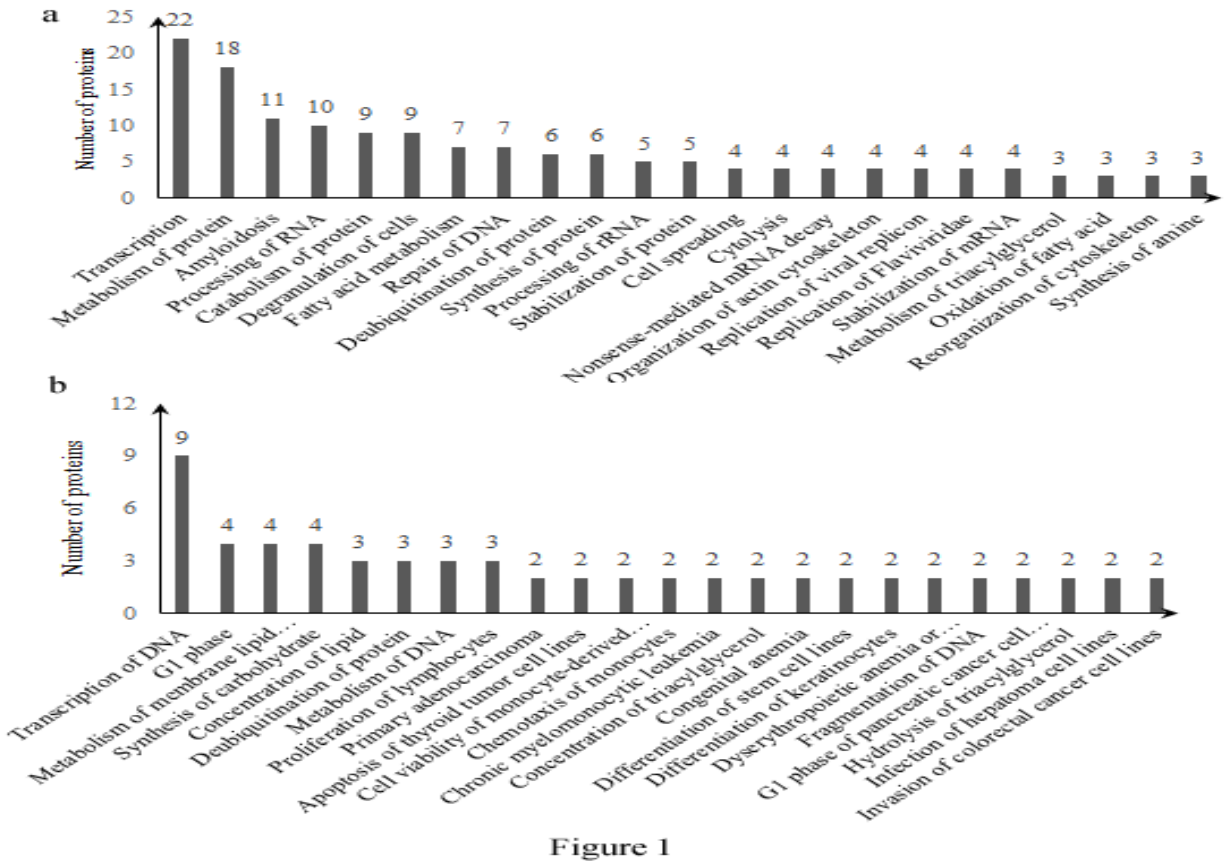

Figure 1

Figure 1. The functional categories of differentially expressed proteins in the glucose, soy oil, and glutamine groups on day 14 (Figure 1.a). The functional categories of differentially expressed proteins in the glucose, soy oil, and glutamine groups on day 28 (Figure 1.b).

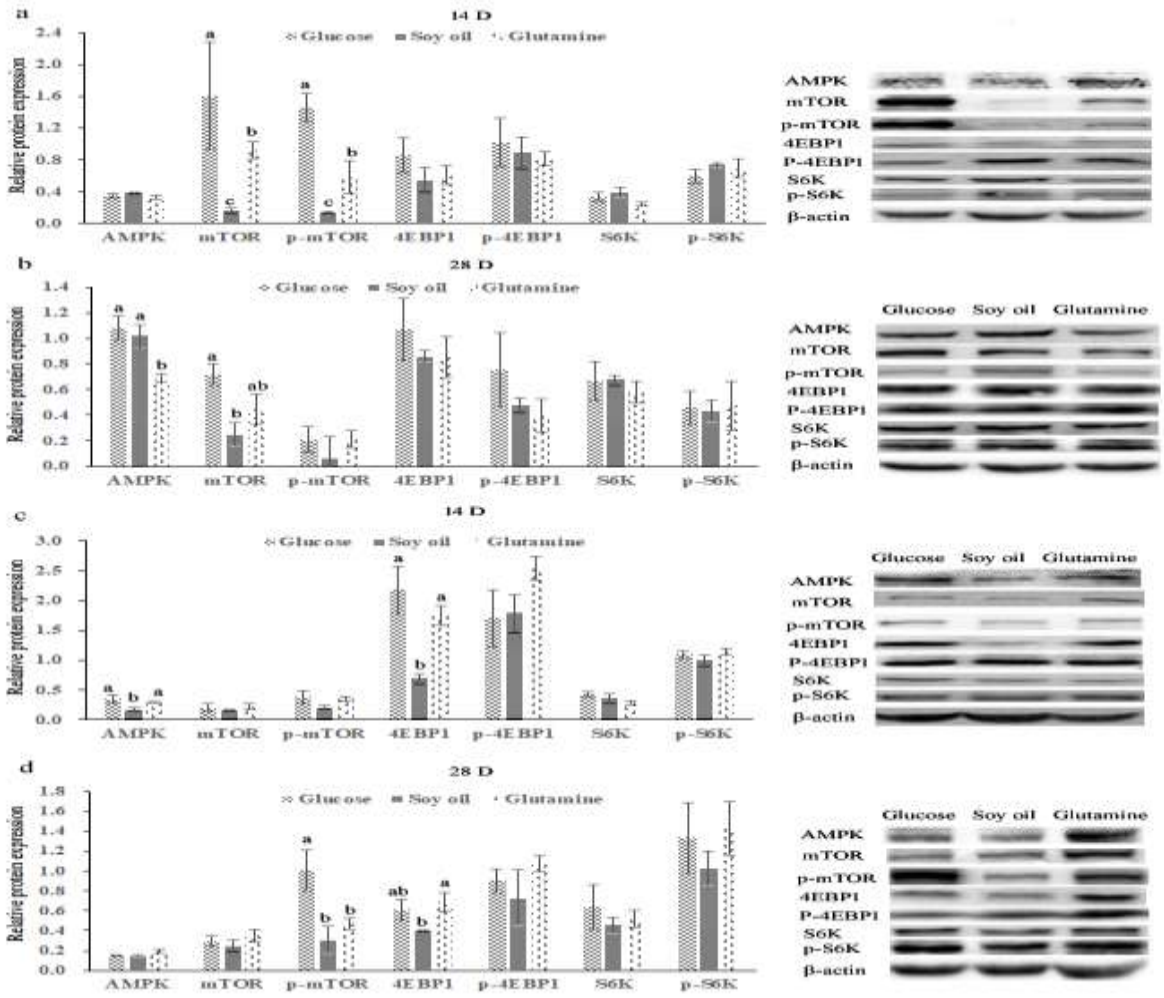

Figure 2. Effects of dietary supplementation of different energy sources on protein expression of the mammalian target of rapamycin complex (mTORC1) signaling pathway in the upper villus (a, b) or crypt cell (c, d) fraction. The relative abundance of proteins in mTOR signaling pathway proteins are based on the expression of these proteins determined by western blot on day 14 or 28. Data are expressed as mean ± SEM. *P* value with different letters within the same organ are different ($P < 0.05$). AMPK, AMP-activated protein kinase; 4EBP1, eukaryotic initiation factor-4E binding protein-1; p-4EBP1, phospho-4EBP1; p-mTOR, phosphor-mTOR; S6K, s6 kinase; p-S6K, phosphor-S6K;

Supplemental Figure legend

Supplemental Figure 1. Representative staining of jejunal mucosal morphology of weaned piglets supplemented with glucose (a, d), soy oil (b, e) or glutamine (c, f) on day 14 and 28.