Glutamine, Glutamate, and Aspartate Improves Morphology and Energy Production of Small Intestine in Piglets with Different Energy Levels Diets

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Abstract

Background: Weaning-stress may cause reduced energy intake for maintenance of mucosal structure. Glutamine (Gln), glutamate (Glu), and aspartate (Asp) are major energy sources for small intestine. This study investigated whether Gln, Glu, and Asp improve the intestinal morphology via regulating the energy metabolism in weaning-piglet. A total of 198 weaned-piglets were assigned to the following treatments: Control (Basal diet + 1.59% L-Alanine); T1 (Basal diet + 1% L-Gln + 0.5% L-Glu + 0.1% L-Asp); T2 (Low energy diet + 1% L-Gln + 0.5% L-Glu + 0.1% L-Asp). Jejunum and ileum were obtained on d-5 or d-21 post-weaning.

Results: Improved growth performance on d-21 post-weaning were observed in T1 treatment. Both T1 and T2 treatments improved small intestinal morphology by increasing villus height, goblet cell number and decreasing crypt depth on d 5 or d-21 post-weaning. Gln, Glu, and Asp could restore the small intestinal energy homeostasis via replenishing the Krebs' cycle and down-regulate the AMPK pathway. However, when piglets fed by a low energy diet, Gln, Glu, and Asp are not sufficient to maintain the intestinal energy balance on d-5 post-weaning so that the AMPK, beta-oxidation, and mitochondrial biogenesis are activated to meet the high energy demand of enterocytes.

Conclusion: These data indicated that Gln, Glu, and Asp could restore the energy homeostasis of intestinal mucosa of piglets. And the mucosal energy metabolism showed different responses to the intervention of Gln, Glu, and Asp in piglets with a low energy diet between d5 and d21 post-weaning. These findings provide new information on the nutritional intervention for the insufficient energy intake in weaning-piglet.

Background

At weaning, young pigs are subjected to removal from sow and littermates and are transported to a new environment, as well as their diet is abrupt changed from sow's milk to solid diet, which cause low voluntary food intake and associated poor intestinal barrier structure and function [1, 2]. Undoubtedly, the weaning pigs are not eating enough food and their intestinal mucosa is failing to digest and absorb sufficient nutrients to cover their energy requirement for maintenance [3], which are major limitations to improve the growth of piglet after-weaning. Therefore, improving the energy intake from exogenous nutrition is rationally served as a promising approach to protect piglets from weaning stress.

The intestine of piglet has notably high energy demand due to the repaid renewal of epithelium within few days [4, 5]. Hence, the epithelial cells of gastrointestinal (GI) tract require intense anabolic metabolism. It has been demonstrated that GI tract represents approximately 5% of body weight, whereas it is responsible for about 20% of whole body O_2 consumption [6]. Glucose and lipid are major sources for the supply and storage of energy in cells [7]. AMP-activated protein kinase (AMPK) is the master regulator of energy metabolism [6]. AMPK increases adenosine triphosphate (ATP) levels by promoting glucose and lipids breakdown and inhibiting their synthesis and storage [7]. Glycolysis, Krebs' cycle, and fatty acids beta-oxidation are the main catabolic processes for glucose and lipids [8], respectively. Although all animals obtain their biological energy from the cell-specific oxidation of fatty acids, glucose, and amino acids in diets [9], for gastrointestinal tract, glutamine (Gln), glutamate (Glu), and aspartate (Asp) are the main energy sources to maintain gut integrity and function [10, 11]. Meanwhile, numerous studies have reported that Gln, Glu, and Asp has profound impacts on the intestinal nutrition and health. Gln plays multiple roles in regulating intestinal protein turnover [12], gene expression [13], cell proliferation [14] and immune function [15]. Asp could improve growth performance of weanling piglets [16], and attenuate the intestinal injury induced by *Escherichia coli* lipopolysaccharide (LPS) [17]. Nevertheless, a typical corn- and soybean meal-based diet cannot provide sufficient amounts of Asp, Glu, and Gln for protein synthesis in post-weaning pigs [18].

In the present study, we hypothesize that Gln, Glu, and Asp could improve the small intestinal structure of weanling piglets via regulating the enterocyte energy metabolism. The effects of supplementation of Gln, Glu, and Asp on the intestinal morphology, energy metabolites, AMPK pathway phosphorylation were determined in weaning piglets on d 5 and d 21 post-weaning.

Methods

All animals used in this study were humanely managed according to the Chinese Guidelines for Animal Welfare. The experimental protocol was approved by the Animal Care and Use Committee of the Chinese Academy of Sciences (Beijing, China).

Animals and experimental design

A total of 198 piglets (Duroc ×Landrace × Large Yorkshire) weaned at 21-d of age were assigned to 18 pens based on their body weight (BW). There were 11 piglets per pen and 6 pens per treatment. The treatment groups include: i) Control (Basal diet + 1.59% L-Alanine (Ala); iso-nitrogenous control); ii) T1 (Basal diet + 1% L-Gln + 0.5% L-Glu + 0.1% L-Asp); iii) T2 (Low energy diet + 1% L-Gln + 0.5% L-Glu + 0.1% L-Asp) (Table 1). The basal diets were formulated to meet the nutrient requirements recommended by the National Research Council (2012). A low energy level diet was obtained by removing the soybean oil, glucose, and sucrose. The dose was based on the growth performance of piglets in the preliminary experiment. All piglets were housed in an environmentally well controlled nursery facility with slatted plastic flooring and a mechanical ventilation system, and had free access to drink water.

One piglet closed to the average body weight from each pen was slaughtered on day 5 (w5d) and 21 (w21d) post-weaning. After being stunned electrically, piglets were sacrificed and the jejunum and ileum was dissected and rinsed thoroughly with ice-cold physiological saline. The middle segments of the jejunum (2 cm) and ileum (2 cm) were cut and fixed in 2.5% glutaraldehyde or 4% formaldehyde for morphological and immunohistochemical analysis. Samples of the jejunal and ileal mucosa were scraped, immediately snap frozen in liquid nitrogen and stored at -80 °C for further analysis.

Intestinal morphology

The segments of the jejunum and ileum fixed in 4 % formaldehyde were used to determine morphology using hematoxylin-eosin staining. After dehydration, embedding, sectioning, and staining, images were acquired at various magnifications with computer-assisted microscopy (Micro-metrics TM; Nikon ECLIPSE E200, Tokyo, Japan). Villous height, crypt depth, goblet cell and lymphocyte counts were measured by Image-Pro Plus software, Version 6.0 on images at 200- or 400-fold magnification in five randomly selected fields, respectively [19].

Segments of the jejunum and ileum at 150-fold magnification were also designated for analysis by scanning electron microscopy as described by German [20] and Liu et al. [21]. Briefly, tissue segments were fixed with 2.5 % glutaraldehyde for 2 h at 4 °C, and rinsed 3×10 min in PBS at 4 °C. The tissues were then postfixed in 1 % osmium tetroxide for 12 h at 4 °C, and rinsed 3×10 min in PBS at 4 °C. After samples were mounted onto stubs by means of quick-drying silver paint, the tissues were coated with gold-palladium and examined by a JEOL JSM-6360LV scanning electron microscope at 25 KV. The apparent characteristics of microvillus were observed and described.

After samples were mounted onto stubs by means of quick-drying silver paint, the tissues were coated with gold-palladium and examined by a JEOL SU8010 scanning electron microscope at 1.0 KV. The apparent characteristics of microvillus were observed and described.

Metabolomic profiling

The ileal mucosal metabolomic profile was analyzed by liquid chromatography (LC)- mass spectrometry (MS)/MS. Briefly, 100 mg ileal mucosa was homogenized in 200 μL ddH_2O, and mixed with 800 μL methanol/acetonitrile (1:1, v/v) and sonicated in ice water-bath for 30 min. After incubation in -20 °C for 1 h, the mixture was centrifuged at 14,000 *g* for 15 min at 4 °C. The supernatant was dried by speedvac, resuspended in 100 μL methanol/ddH_2O (1:1, v/v) for LC-MS/MS analysis. An aliquot from each sample was pooled to create quality control (QC) samples that were used to evaluate the internal standards and instrument performance. The ultra-high-performance LC (UPLC; Agilent 1290 Infinity LC, USA) was performed on a 2.1 x 100 mm ACQUITY UPLC BEH Amide column (internal diameter 1.7 μm; Waters, USA). The column was warmed to 45 °C before use. The mobile phase for UPLC analysis consisted of two solutions: (A) 15 mM ammonium acetate/H_2O and (B) acetonitrile. The

MS/MS (5500 QTRAP, AB SCIEX, USA) spectra was set as follows: source temperature 450 °C, ion Source Gas 1: 45, Ion Source Gas 2: 45, Curtain gas: 30. ionSapary Voltage Floating-4500 V.

Real-Time Quantitative Reverse Transcriptase PCR

Expressions of pyruvate dehydrogenase kinase (*Pdk4*), phosphoenolpyruvate carboxykinase 1 (*Pck1*), succinate dehydrogenase 1 (*Sdh1*), mitochondrial uncoupling protein 2 (*Ucp2*), peroxisome proliferator-activated receptor alpha 1 (*Pparα1*), peroxisomal acyl-coenzyme A oxidase 1 (*Acox1*), carnitine palmitoyltransferase 1A (*Cpt1a*), acetyl-CoA carboxylase (*Acc*), *Ampk*, Sirtuin 1 (*Sirt1*), peroxisome proliferator-activated receptor gamma coactivator 1-α (*Pgc1α*), mitochondrial transcription factor A (*Tfam*), and nuclear respiratory factor 1 (*Nrf1*) mRNA in jejunal and ileal mucosa were determined by real-time quantitative reverse transcriptase PCR (real-time qRT-PCR) as described previously [22]. Primers were designed with Primer 5.0 (PREMIER Biosoft International, Palo Alto, CA) according to the gene sequence of the pig to produce an amplification product (Table S1). The comparative threshold cycle (Ct) value method ($2^{-\Delta\Delta Ct}$) was employed to quantitate expression levels for target genes relative to those for the β-Actin. Data were expressed as the relative values to those of control piglets.

Western blotting

Jejunal and ileal mucosa samples were homogenized, and protein concentrations were measured using the bicinchoninic acid assay method with BSA as standard (Beyotime Institute of Biotechnology, Shanghai, China). All samples were adjusted to an equal concentration (70 μg protein). The supernatant fluid (containing tissue proteins) was diluted with 5 × sodium dodecyl sulfate (SDS) sample buffer and heated in boiling water for 5 min and cooled down for Western blot analysis [23]. The following first antibodies were used for protein quantification: AMPKα (1:1,000; Cell Signaling Technology, USA), phosphorylated AMPKα (p-AMPK (Thr175); 1:1,000; Cell Signaling Technology, USA), ACC (1:1,000; Cell Signaling Technology, US), phosphorylated ACC (p-ACC (Ser79); 1:1,000; Cell Signaling Technology, USA), SirT1 (1:1,000; Cell Signaling Technology, USA), PGC1α (1:1,000; Abcam, UK) and β-actin (1:1000; Cell Signaling Technology, USA) as well as secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit IgG; 1:5000; Boster Biological Technology, Wuhan, China). The images were detected by chemiluminescence (Millipore, Billerica, MA). Each Western blot was subjected to multiple exposures to ensure that the chemiluminescence signals were linear. Western blots were quantified by measuring the intensity of correctly sized bands using software (Alpha Imager 2200 Software; Alpha Innotech Corporation, San Leandro, CA, USA) and protein measurement was normalized to β-actin.

Statistical analysis

The growth performance was performed by one-way ANOVA using SPSS software 19.0 (SPSS Inc., Chicago, IL). The differences among treatments were evaluated using Tukey's test.

The intestinal morphology, metabolomic profiles, gene and protein expression were analyzed using the general linear models' procedure, with diet treatments, days after weaning, and the interaction between treatment and day as the main effects. The results in the tables were presented as main effects. When no significant interaction between treatment and day was found, the differences among treatments or days were evaluated using Tukey HSD test (showed in Table 3, 4, 5, 6). When significant interaction between treatment and day was found, a simple main effect analysis would be performed and the differences among 3 treatments across 2 levels of days post-weaning were evaluated using Bonferroni test (showed in Figure 1, 2, 3). The developmental changes of the intestine were not the main outcome, so the differences between d 5 and d 21 post-weaning across 3 levels of treatments were not showed. Differences were declared as significant at $P < 0.05$.

Results

Growth performance

Initial body weight (BW), final BW, average daily gain (ADG), average daily feed intake (ADFI), and the ratio of average daily feed intake to average daily gain (F / G) were showed in Table 2. The initial BW was similar among treatments ($P = 0.959$). On d 5

post-weaning, there were no differences on BW (P = 0.952), ADG (P = 0.782), ADFI (P = 0.127) and F/G (P = 0.240) among different treatments. On d 21 post-weaning, basal dietary supplementation with Gln, Glu, Asp (T1) improved the final BW and ADG of piglets in comparison to those in control group (P < 0.05). However, the digestible energy intakes of weanling piglets neither on d 5 (P = 0.190) nor d 21 (P = 0.709) post-weaning were not significantly different.

Jejunal and ileal morphology

In Figure 1A and B, the scanning electron microscopy showed that the villi in the jejunum and ileum on d 5 post-weaning were thin and sparse, and then grew shorter, stouter and denser on d 21 post-weaning. In the jejunum, the villi heights among control, T1, and T2 groups were not showed significantly different on w 5 d, but there was an increase in villi density and villi-fold in T1 and T2 group on w 21 d. In the ileum, the increased villi densities were observed in control and T1 groups in comparison to T2 group on w 5 d, whereas villi in T1 and T2 group were denser and stouter than villi in control group on w 21 d.

The main effects of diet treatments and days after weaning on parameters from the intestinal hematoxylin-eosin staining were showed in Table 3. When significant interactions between treatments and days after weaning were obtained, differences among control, T1 and T2 on d 5 or d 21 post-weaning were showed in Figure 1C. In Table 3, the number of goblet cells in the jejunum of T2 group was higher than these in T1 group (P < 0.05). There were interactions (P < 0.05) between treatments and days on the jejunal villus height, ileal villus height and crypt depth, which means days after weaning influenced the treatments' efficacy. In Figure 1C, a low energy diet supplemented with Glu, Gln, and Asp increased (P < 0.05) the villus height of jejunum on d 21 post-weaning and decreased (P < 0.05) the crypt depth of ileum on d 5 post-weaning as compared to the control group, whereas it had lower (P < 0.05) ileal villus height than the control and T1 groups on d 5 post-weaning. A basal diet supplemented with Gln, Glu, and Asp increased the villus height of ileum on d 21 post-weaning compared to the control group.

Metabolomic profiling of jejunal mucosa of weanling piglets

The jejunal mucosa energy metabolite fingerprints were presented in Figure 2A and Figure 2B. Piglets on d 5 and d 21 postweaning could be readily differentiated by the heat map of their jejunal mucosa energy metabolites (Figure 2A). Piglets on d 5 post-weaning showed more abundances of dihydroxyacetone phosphate (DHAP), isocitrate, aconitate, citrate, guanosine 5 monophosphate (GMP), fructose 6 phosphate (F-6-P), and glucose-6-phosphate (G-6-P) in jejunal mucosa, while piglets on d 21 post-weaning showed more abundances of fumarate, malate, flavin mononucleotide (FMN), cyclic- adenosine 5 monophosphate (c-AMP), AMP, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), nicotinamide adenine dinucleotide phosphate ($NADP^+$), nicotinamide adenine dinucleotide (NAD^+), adenosine 5 diphosphate (ADP), and thiamine pyrophosphate(TPP) in jejunal mucosa. The first two components of a principal component analysis (PCA) showed that the jejunal mucosal energy metabolites of control and T1 treatments on d 5 post-weaning were comparable, while T2 treatment on d 5 post-weaning was more dispersive and different from control and T1 treatments. On d 21 post-weaning, energy metabolites in T1 and T2 treatments were comparable, which were different from these in the control group.

The main effects of diet treatments and days after weaning on metabolites' levels of jejunal mucosa were showed in Table S2. When significant interactions between treatments and days after weaning were found, differences among control, T1 and T2 on d 5 or d 21 post-weaning were showed in Figure 2C. In Table S2, supplementation with Gln, Glu, and Asp in both basal and low energy diets increased (P < 0.05) the succinate level but decreased (P < 0.05) the phosphoenolpyruvate level in jejunal mucosa of weanling piglets. Days post-weaning had influences on the diet treatments' efficacy ($P_{interaction}$ < 0.05). As shown in Figure 2C, a basal diet supplemented with Gln, Glu, and Asp (T1) increased (P < 0.05) the pyruvate, α-ketoglutarate (AKG), isocitrate, and fructose 1,6 phosphate (F-1,6-P) levels but decreased (P < 0.05) the NADPH level on d 5 post-weaning compared to control group. A low energy diet supplemented with Gln, Glu, and Asp (T2) increased (P < 0.05) the pyruvate, AKG, isocitrate and GMP levels compared to the control group and increased (P < 0.05) DHAP, NADPH, and AMP levels as compared to control and T1 groups but reduced (P < 0.05) 3-phosphoglycerate, F-6-P, and G-6-P levels on d 5 post-weaning. On d 21 post-weaning, T1 and T2 treatments showed higher (P < 0.05) oxaloacetate level than the control group.

Expression of gene involved in energy metabolism of jejunal and ileal mucosa of piglets

The gene expressions of several key enzymes of energy metabolism were presented in Table S3 and Figure 2D. The main effect in Table S3 showed T1 treatment increased ($P < 0.05$) the ileal *Ucp2* mRNA level compared to the control group but decreased ($P < 0.05$) the ileal *Cpt1a* mRNA level compared to control and T2 groups. T2 treatment increased ($P < 0.05$) the jejunal *Acox1* and ileal *Cpt1a* mRNA levels in comparison with the control group. Days post-weaning had influences on the diet treatments' efficacy ($P_{interaction} < 0.05$; Figure 2D). On d 5 post-weaning, a low energy diet supplemented with Gln, Glu, and Asp had lower ($P < 0.05$) *Pdk4* and *Pck1*mRNA abundances than control or T1 groups. On d 21 post-weaning, a basal diet supplemented with Gln, Glu, and Asp enhanced ($P < 0.05$) the *Sdh1* mRNA abundance in comparison to these in the control group. A low energy diet supplemented with Gln, Glu, and Asp increased ($P < 0.05$) the *Pck1* and *Sdh1* mRNA abundances as compared to T1 or control groups, respectively.

mRNA and protein abundances of AMP-activated protein kinase signaling pathway in jejunal and ileal mucosa of piglets

The relative mRNA and protein abundances of AMPK pathway in the jejunal and ileal mucosa were presented in Table S4, S5 and Figure 3, respectively. The main effects on AMPK pathway gene expressions in Table S4 showed a basal diet supplemented with Gln, Glu and Asp (T1) lowered ($P < 0.05$) jejunal *Pgc1α*, *Tfam*, and *Nrf1* and ileal *Sirt1*, *Pgc1α* mRNA levels compared to control and T2 group, as well as ileal *Tfam* mRNA level compared to the T2 group. The main effects on AMPK pathway protein abundances in Table S5 showed T1 treatment enhanced ($P < 0.05$) ileal ACC protein abundance as compared to that in the control group. Days post-weaning did not affect the diet treatments' efficacy on the AMPK pathway gene expression ($P_{interaction} > 0.05$) but had influenced the treatments' efficacy on AMPK pathway protein abundances ($P_{interaction} < 0.05$). As shown in Figure 3, a basal diet supplemented with Gln, Glu and Asp (T1) enhanced ($P < 0.05$) the jejunal AMPKα protein abundance compared to the control group but reduced ($P < 0.05$) the ileal p-AMPKα and PGC1α protein abundances as compared to control and T2 groups, respectively, on d 5 post-weaning. A low energy diet supplemented with Gln, Glu and Asp (T2) reduced ($P < 0.05$) the ileal AMPKα protein abundance compared to control and T1 groups, while increased ($P < 0.05$) jejunal and ileal SirT1 and PGC1α, as well as jejunal ACC and p-ACC protein abundances compared to control or T1 groups on d 5 post-weaning. On d 21 post-weaning, T1 and T2 treatments lowered ($P < 0.05$) the jejunal ACC, ileal AMPKα and PGC1α, as well as jejunal and ileal p-AMPKα, p-ACC, and SirT1 protein abundances as compared to the control groups. Meanwhile, T1 treatment showed lower ($P < 0.05$) jejunal and ileal PGC1α and ileal SirT1 protein abundances than these in the T2 group.

Discussion

The mammalian intestine plays a key role in nutrients intake and has a high rate of energy expenditure because the digestion and absorption processes are highly dependent on energy [24, 25].Weaning results in a growth check caused by a period of low feed intake, which also reduces the energy intake of intestine of piglets [26, 27]. Successful adaptation to these changes requires profound morphological and energy metabolic adjustments of the gastrointestinal tract [28]. Gln, Glu and Asp were used to help the faster restitution of mucosal barrier after stress injury, and to improve the intestinal energy metabolism of piglets with different energy levels diets in this study.

The most serious damages in piglets during weaning-stress occurs on d 2 to d 5 post-weaning [25, 29], and is usually associated with a dramatic reduction of feed intake resulting in marked changes in the growth pattern on body weight [28]. In the present study, supplementation with 1 % Gln, 0.5 % Glu, and 0.1 % Asp in a normal energy level diet enhanced the BW and ADG on d 21 post-weaning. The beneficial effect of Gln, Glu and Asp on improving the growth-suppression induced by weaning stress in our study was consistent with previous researches, which have shown that Glu and Asp exert positive effects on growth performance of animals in healthy and oxidative-stress conditions [30, 31] [32, 33]. Although low energy diet did not significantly increase the ADFI of weanling piglets, the digestive energy intake in T2 group was comparable with these in control and T1 groups. Piglets in low energy diet treatment increased their satiety through increasing food intake.

In addition to poor growth performance, the growth-check induced by weaning-stress is accompanied by impaired mucosal integrity, villous atrophy, an increased rate of cell loss, or a decreased rate of crypt-cell production [34, 35]. Supplementation with Gln, Glu, and Asp in normal energy diet increased ileal villus height on d 21 post-weaning. The major reasons for villus atrophy are deficient enteral nutrition and reduced energy intake independent of diet type [36, 37]. We found that a low energy diet

supplemented with Gln, Glu, and Asp could alleviate impaired intestinal structure by raising jejunal villus height and goblet cells number or reducing ileal crypt depth on either d 5 or d 21 post-weaning. The critical roles of these three amino acids in intestine have been reported in many well-designed studies, for example, Gln could increase the villus length of jejunum [38], and Asp could alleviate the intestinal barrier damage [32, 39, 40]. The results in our current study indicated that Gln, Glu, and Asp could improve the intestinal barrier structure of weanling piglets fed either a low energy diet or a normal energy diet, which led us to further explore the mechanism of these three amino acids regulating energy homeostasis in the small intestine.

ATP production and utilization are very active in pig enterocytes. The relative abundances of intermediate metabolites involved in the energy metabolism of the small intestine of piglets on d 5 and d 21 post-weaning were not comparable, and the regulation of Gln, Glu, and Asp on energy metabolites varied between d 5 and d 21 post-weaning. It may indicate that the energy metabolism in the small intestine changes during the weaning period [41], and the pattern of metabolism has different responses to amino acids. It is well established that dietary Gln, Glu, and Asp plus arterial Gln provide up to 80 % of ATP to the small intestine mucosa in mammals [11, 42, 43]. After these three amino acids are absorbed into enterocytes, they could be metabolized and oxidized via the Krebs' cycle to water and CO2 yielding ATP [44]. The oxidative steps of Gln and Glu in pig enterocytes are similar. Gln firstly enter the mitochondrial in order to be degraded to Glu and ammonia by the mitochondrial glutaminase [45]. Glu could be transaminated to produce AKG, which is the key molecular of the Krebs' cycle [46]. Asp replenished the Krebs cycle by being converted to oxaloacetate [44]. In the present study, supplementation with Gln, Glu, and Asp either in a regular energy diet or a low energy diet could replenish the jejunal Krebs' cycle, manifested as increased pyruvate, oxaloacetate, isocitrate, AKG, and succinate levels on d 5 or d 21 post-weaning. Meanwhile, supplementation with Gln, Glu and Asp in either regular energy diet or low energy diet enhanced *Sdh1* mRNA abundance on d 21 post-weaning, whereas low energy diet supplemented with Gln, Glu, and Asp declined *Pdk4* mRNA abundance on d 5 post-weaning. SDH1 is part of Krebs' cycle and oxidizes succinate to fumarate [47]. The higher Sdh1 expression was consistent with the higher succinate level in the jejunal mucosa. *Sdh1* is responsible to promote the oxidation of succinate and prevents the accumulation of succinate in the mitochondria [48].PDK4 is a regulator of pyruvate dehydrogenase (PDH), as it could inactivate PDH by phosphorylation [49]. PDH represents a cornerstone in cellular energy metabolism, linking glycolysis and Krebs' cycle and lipid metabolism [50]. In this study, PDH activity was failed to detected, whereas the decreased *Pdk4* gene expression might suggest the influx of acetyl-CoA from glycolysis into the Krebs' cycle increased in response to T2 treatment on d 5 post-weaning. Indeed, a low energy diet addition with Gln, Glu, and Asp (T2) showed lower G-6-P, F-6-P, F-1,6-P, and 3-phosphoglycerate, but higher DHAP in jejunal mucosa on d 5 post-weaning, whereas the T2 did not affect these intermediate metabolites on d 21 post-weaning. Nevertheless, a normal energy diet addition with Gln, Glu, and Asp had no effect on the levels of glycolytic metabolites on neither d 5 nor d 21 post-weaning, except for the increased F-1,6-P levels on d 5 post-weaning. Our results suggested that dietary supplementation with Gln, Glu, and Asp in either a normal energy diet or a low energy diet can replenish the Krebs' cycle in enterocyte by converting to the intermediate metabolites. However, a low energy diet supplementation with Glu, Gln, and Asp seemed to promote glycolytic influx on d 5 instead of d 21 post-weaning. Unlike the low diet treatment, a normal diet addition with Gln, Glu, and Asp did not significantly affect the glycolysis during the weaning-period.

Fatty acids oxidation also plays critical role in energy homeostasis [51]. UCP2 is involved in the regulation of mitochondrial substrate oxidation, which could be stimulated by fatty acid and glutamine [52, 53]. Several studies suggested that the role of UCP2 would be to promote oxidation of glutamine and fatty acids rather than that of the pyruvate derived from glucose [54, 55]. Indeed, a normal energy diet supplementation with Gln, Glu, and Asp raised the *Ucp2* mRNA abundance but decreased *Cpt1a* in ileal mucosa. Although a low energy diet supplementation with Gln, Glu, and Asp did not affect the *Ucp2* gene expression, it upregulated the jejunal mucosal *Acox1* and ileal mucosal *Cpt1a* mRNA abundances. CPT1 and Acox1 play key roles in the fatty acid beta-oxidation in either mitochondria or peroxisomes, respectively. CPT1 is the key enzyme in the carnitine-dependent transport across the mitochondrial inner membrane [56], while Acox1 is the first enzyme of the fatty acid beta-oxidation in peroxisomes, which catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs [57]. Based on these results, it may be suggested that a low energy diet addition with Gln, Glu, and Asp activated the fatty acids beta-oxidation. Besides, supplementation with Gln, Glu, and Asp in a low energy diet increased jejunal mucosal NADPH, GMP and AMP levels, as well as the *Pck1* gene expression on d 5 post-weaning. NADPH is mainly produced through pentose cycle, which is an alternative way of glucose use [58]. Because the current study did not detect other enzymes or intermediate metabolites involved in the pentose

cycle, it is not clear whether a low energy diet supplementation with Gln, Glu, and Asp regulates the NADPH levels via the pentose cycle. Moreover, the decreased *Pck1* gene expression, which is a main control point of the regulation of gluconeogenesis [59], may implicate the reduced gluconeogenesis in the T2 group on d 5 post-weaning. The higher AMP and GMP levels activates the AMPK signaling pathway, which is a key sensor of energy balance responding to low energy status and upregulating catabolic pathways and downregulating anabolic pathways [60]. Thus, it is reasonable to speculate that a low diet addition with Gln, Glu, and Asp activates the AMPK signaling pathway.

A low energy diet supplementation with Gln, Glu, and Asp (T2) up-regulated the AMPK and ACC phosphorylation, as well as the SirT1 and PGC1α protein abundance in the small intestine on d 5 post-weaning, whereas it down-regulated the AMPK pathway phosphorylation levels on d 21 post-weaning. Under conditions of low energy, activated AMPK phosphorylates downstream substrates, increasing ATP levels by promoting glucose and lipids breakdown and inhibiting their synthesis and storage [7, 61]. This is consistent with the observation from energy metabolites and these enzymes' expression mentioned above. AMPK could acutely regulate glycolysis and reduce gluconeogenesis, and controls overall lipids metabolism through direct phosphorylation of ACC and simultaneously promoting fatty acid oxidation by relieving the suppression of CPT1 [7]. Another interesting finding is that the PGC1*α* gene and protein abundances were enhanced by a low energy diet supplementation with Gln, Glu, and Asp (T2). PGC1*α* provides a direct link between external physiological stimuli and the regulation of mitochondrial biofunction [62]. PGC1*α* regulates mitochondrial biogenesis via regulation of Nrf1 [63], which in turn activates TFAM that involved in the mDNA replication [6]. In the current study, the increased *Nrf1* and *Tfam* gene expression in T2 treatment suggested that a low energy diet addition with Gln, Glu, and Asp stimulated the mitochondrial biogenesis in response to the acute energy crises. Unlike the low energy diet treatment, supplementation with the amino acids in a normal diet did not affect the AMPK system on d 5 post-weaning while down-regulated the phosphorylated AMPK pathway levels on d 21-post weaning. Previous studies have demonstrated that Gln could regulate enterocyte growth under normal energy conditions through activating mTOR pathway but inhibiting AMPK pathway [64, 65]. Days 2 to 5 after weaning is the most severe stage during the weaning-period [22]. Piglets on d 2 to 5 post-weaning requires more energy to rebuild the impaired intestinal mucosal barrier [66]. It is possible that Gln, Glu, and Asp could restore the small intestinal energy homeostasis via replenishing Krebs' cycle under a normal energy diet condition, whereas it cannot provide sufficient energy sources to piglets fed with a low energy diet so that the AMPK pathway and mitochondrial biogenesis are activated by low energy conditions. While on d 21 post-weaning, the remodeling of the intestinal mucosa has been achieved, and the digestive system has adapted to utilize solid feed components to absorb and transport more energy sources [67, 68], Gln, Glu, and Asp could balance the intestinal energy homeostasis in piglets fed with a low energy diet.

Conclusions

The present result shows that supplementation of Gln, Glu, and Asp in a normal energy diet can promote the growth performance of weaning-piglet on d 21 post-weaning. Supplementation with Gln, Glu, and Asp in either a normal energy diet or a low energy diet improved the small intestinal mucosal barrier structure by raising jejunal villus height and goblet cells number or reducing ileal crypt depth on d 5 or 21 post-weaning. In general, Gln, Glu, and Asp could restore the small intestinal energy homeostasis via replenishing the Krebs' cycle and down-regulate the AMPK pathway. However, when piglets under specific conditions, such as energy crisis and severe weaning-stress challenge, Gln, Glu, and Asp are not sufficient to maintain the intestinal energy balance so that the AMPK, fatty acids beta-oxidation, and mitochondrial biogenesis pathways are activated to meet the high energy demand of enterocytes of piglets. Our results aid in providing new information about the nutritional intervention of Gln, Glu and Asp for the insufficient energy intake in weaning-piglet.

Abbreviations

Gln: glutamine; Glu: glutamate; Asp: aspartate; AMPK: AMP-activated protein kinase; GI: gastrointestinal tract; ATP: adenosine triphosphate; LPS: lipopolysaccharide; BW: body weight; LC-MS: liquid chromatography - mass spectrometry; QC: quality control; Pdk4: pyruvate dehydrogenase kinase; Pck1: phosphoenolpyruvate carboxykinase 1; Sdh1: succinate dehydrogenase 1; Ucp2: mitochondrial uncoupling protein 2; Pparα1: peroxisome proliferator-activated receptor alpha 1; Acox1: peroxisomal acyl-

coenzyme A oxidase 1; Cpt1b: carnitine palmitoyltransferase 1A; Acc: acetyl-CoA carboxylase; Sirt1: Sirtuin 1; Pgc1α: peroxisome proliferator-activated receptor gamma coactivator 1-α; Tfam: mitochondrial transcription factor A; Nrf1: nuclear respiratory factor 1; qRT-PCR: quantitative reverse transcriptase PCR; ADG: average daily gain; ADFI: average daily feed intake; DHAP: dihydroxyacetone phosphate; GMP: guanosine 5 monophosphate; F-6-P: fructose 6 phosphate; G-6-P: glucose-6-phosphate; FMN: flavin mononucleotide; c-AMP: cyclic- adenosine 5 monophosphate; NADPH: nicotinamide adenine dinucleotide phosphate hydrogen; NAD^+: nicotinamide adenine dinucleotide; $NADP^+$: nicotinamide adenine dinucleotide phosphate; ADP: adenosine 5 diphosphate; TPP: thiamine pyrophosphate; PCA: principal component analysis.

Declarations

Availability of data and materials

The datasets generated or analyzed during this study are presented in this manuscript and available to readers.

Ethics approval

All animals used in this study were humanely managed according to the Chinese Guidelines for Animal Welfare. The experimental protocol was approved by the Animal Care and Use Committee of the Chinese Academy of Sciences (Beijing, China).

Conflicts of interest

There are no conflicts of interest to declare.

Consent for publication

Not applicable.

Author contributions

Bie Tan: Conceptualization, Methodology, Supervision. **Yulong Yin:** Conceptualization. **Jing Wang:** Investigation, Data curation, Formal analysis, Writing-Original draft preparation. **Nan Wang:** Investigation, Data curation. **Ming Qi:** Investigation, Data curation. **Jianjun Li:** Investigation, Data curation, Software.

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Tables

Table 1. Ingredient composition of diets [1]

Item	Control diet	T 1 diet	T 2 diet
Ingredient (%)			
Corn	23.93	24.00	24.40
Extruded corn	35.00	35.00	35.00
Soybean	8.00	8.00	11.80
Fermented soybean	9.00	9.00	4.00
Extruded soybean	0.00	0.00	2.80
Whey powder	6.00	6.00	6.00
Fish meal	4.00	4.00	4.00
Plasma protein powder	2.00	2.00	2.00
Soybean oil	1.00	1.00	0.00
Glucose	3.00	3.00	0.00
Sucrose	2.00	2.00	0.00
L-lysine (98 %)	0.40	0.40	0.40
DL- methionine	0.11	0.11	0.11
L- threonine	0.12	0.12	0.12
L-alanine	1.59	0.00	0.00
L-glutamine	0.00	1.00	1.00
L-glutamate	0.00	0.50	0.50
L-aspartate	0.00	0.10	0.10
Carrier	0.90	0.82	0.82
Organic acid calcium	0.60	0.60	0.60
$CaHPO_4$	1.00	1.00	1.00
Choline chloride (50 %)	0.01	0.01	0.01
Antioxidant	0.05	0.05	0.05
Mineral premix [2]	0.15	0.15	0.15
Vitamin premix [3]	0.04	0.04	0.04
ZnO	0.40	0.40	0.40
Acidifier	0.70	0.70	0.70
Total	100	100	100
Nutrient composition [*]			
Digestible energy (K cal / Kg)	3445.60	3444.56	3227.00
Crude protein	19.57	19.53	19.53
Calcium	0.47	0.47	0.47
Total phosphorus	0.40	0.40	0.40

Total lysine	1.14	1.14	1.11

[1] T 1 = Basal diet containing 1 % L-glutamine, 0.5 % L-glutamate, and 0.1 % L-aspartate; T 2 = energy deficiency diet containing 1 % L-glutamine, 0.5 % L-glutamate, and 0.1 % L-aspartate; the premix did not contain additional copper, zinc, antibiotics, or probiotics.

[2] Mineral premix provided for 1 kg completed diet: Zn (ZnO), 50 mg; Cu (CuSO4), 20 mg; Mn (MnO), 55 mg; Fe (FeSO4), 100 mg; I (KI), 1 mg; Co (CoSO4), 2 mg; Se (Na2SeO3), 0.3 mg.

[3] Vitamin premix provided for 1 kg completed diet: vitamin A, 8,255 IU; vitamin D3, 2,000 IU; vitamin E, 40 IU; vitamin B1, 2 mg; vitamin B2, 4 mg; pantothenic acid, 15 mg; vitamin B6, 10 mg; vitamin B12, 0.05 mg; nicotinic acid, 30 mg; folic acid, 2 mg; vitamin K3, 1.5 mg; biotin, 0.2 mg; choline chloride, 800 mg; and vitamin C, 100 mg.

[*] Calculated.

Table 2. Growth performance of weaning piglets[1,2].

Items	Treatments			SEM	*P*-value
	Control	T 1	T 2		
Days 5 post-weaning					
Initial weight (kg)	7.09	7.05	7.09	0.070	0.959
BW on w5d (kg)	6.89	6.91	6.94	0.063	0.952
ADG (g/pig per day)	-51.29	-34.17	-38.83	9.767	0.782
ADFI (g/pig per day)	69.14	89.20	93.87	5.140	0.127
F/G	-1.35	-2.61	-2.42	0.893	0.240
Digestible energy intake (K cal/pig)	238.22	307.27	302.91	16.61	0.190
Days 21 post-weaning					
BW on w21d(kg)	11.58^{b}	13.17^{a}	12.07^{ab}	0.278	0.047
ADG (g/pig per day)	224.57^{b}	306.00^{a}	248.88^{ab}	13.801	0.035
ADFI (g/pig per day)	477.42	526.72	524.54	25.139	0.691
F/G	2.13	1.72	2.11	0.136	0.405
Digestible energy intake (K cal/pig)	1644.99	1814.33	1692.69	83.900	0.709

[1] Control = basal diets containing 1.59 % L-alanine (iso-nitrogen); T 1 = basal diets containing 1 % glutamine, 0.5 % glutamate, 0.1 % aspartate; T 2 = energy deficiency diets containing 1 % glutamine, 0.5 % glutamate, 0.1 % aspartate; w 5 d = days 5 post-weaning at d 21 of age, w 21 d = days 21 post-weaning at d 21 of age; BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; F / G = gain: feed ratio.

[2] [a-c] Values with different letters within the same row are different ($P < 0.05$); n = 6.

Table 3. Jejunal and ileal morphology of weanling piglets [1, 2, 3].

Items	Diet treatments			Days post-weaning		*SEM*		*P*-value		
	Control	T 1	T 2	5	21	Diet	Day	Diet	Day	Diet × Day
Jejunum										
Villus height (μm)	375.31	393.77	392.16	337.64	436.52	10.44	8.52	0.394	<0.001	0.049
Crypt depth (μm)	119.98	117.11	118.58	112.95	124.16	5.15	4.20	0.925	0.069	0.284
VCR	3.15	3.43	3.36	3.02	3.61	0.17	0.14	0.483	0.006	0.815
Goblet cells	10.68^{ab}	10.23^{b}	11.17^{a}	10.42	10.97	0.25	0.20	0.044	0.069	0.503
Lymphocyte cells	38.98	38.7	39.62	39.73	38.47	0.58	0.47	0.528	0.069	0.626
Ileum										
Villus height (μm)	358.17	390.65	347.49	322.49	408.39	10.21	8.34	0.015	<0.001	0.020
Crypt depth (μm)	118.32	117.98	106.56	101.34	127.23	3.48	2.84	0.037	<0.001	0.046
VCR	3.05	3.37	3.26	3.22	3.23	0.13	0.10	0.203	0.936	0.634
Goblet cells	10.53	10.82	10.72	10.53	10.84	0.32	0.26	0.816	0.403	0.455
Lymphocyte cells	40.12	40.67	39.65	40.03	40.26	0.38	0.31	0.189	0.619	0.483

[1] Control = basal diets containing 1.59 % L-alanine (iso-nitrogen); T 1 = basal diets containing 1 % glutamine, 0.5 % glutamate, 0.1 % aspartate; T 2 = energy deficiency diets containing 1 % glutamine, 0.5 % glutamate, 0.1 % aspartate; w 5 d = days 5 post-weaning at d 21 of age, w 21 d = days 21 post-weaning at d 21 of age; VCR = the ratio of villus height to crypt depth.

[2] The unit of goblet cells and lymphocyte is cell number per area.

[3] The results were presented as main effects, and the differences among treatments or days were evaluated using Tukey HSD test. [a-b] Values with different letters within the same row are different ($P < 0.05$); n = 6.

Table S1. Primers used for real-time quantitative reverse transcription-PCR [1].

Gene	Accession No.	Primers
β-Actin	XM_003124280.3	F: 5′-GGATGCAGAAGGAGATCACG-3′
		R: 5′-ATCTGCTGGAAGGTGGACAG-3′
Pdk4	NM_001159306.1	F: 5′-CTACAGCACCAACACCTGTGA-3′
		R: 5′-CATCCGTTCCATATCCTGGCA-3′
Pck1	XM_005673043.3	F: 5′-TCTGGTGTACGAGGCTCTCA-3′
		R: 5′-TTGCCGAAGTTGTAGCCGAA-3′
Sdh1	NM_001179714.1	F: 5′-CCGCTGGTCTCTTCCTATGG-3′
		R: 5′-TGGCCCTAAACACAGGGACT-3′
Ucp2	NM_214289.1	F: 5′-CTCACCCAATGTCGCTCGTA-3′
		R: 5′-ATCTCGTCTTGACCACGTCC-3′
Pparα 1	NM_001044526.1	F: 5′-GCAGATCTCAAGTCTCTCGCC-3′
		R: 5′-CCTTGTTCTGGATGCCGTTG-3′
Acox1	NM_001101028.1	F: 5′-TGTCAACTTCACCCCAACCTG -3′
		R: 5′-AGGTAGGACACCATGCCACA -3′
Cpt1α	NM_001129805.1	F: 5′-GCATTTGTCCCATCTTTCGT -3′
		R: 5′-GCACTGGTCCTTCTGGGATA -3′
Ampk	XM_021076522.1	F: 5′-CTGGATTATGAGTGGAAGGT-3′
		R: 5′-AAGTCAACAGGAGAAGAGTC-3′
Acc	NM_001114269.1	F: 5′-GCCGAAACATCTCTGGGATA -3′
		R: 5′-CTCCAGGACAGCACAGATCA -3′
Sirt1	NM_001145750.2	F: 5′-AACATGAAGAGGTGTGGGTG -3′
		R: 5′-GAACAGGTTGCGGGAATC -3′
Pgc1α	XM_005665970.3	F: 5′-GTGAGACAGAGCCAGGTA-3′
		R: 5′-TTGATGATGAAGGTGAAGGT-3′
Tfam	NM_001130211.1	F: 5′-CCACCCTGAGTGGTTTTCCA-3′
		R: 5′-TGCCAGTCTGCCCTATAAGC-3′
Nrf1	XM_021078993.1	F: 5′-CATGGCACTCAACAGCGAAG-3′
		R: 5′-ACATGCTCACAGGGATCTGG-3′

[1] Pdk4: pyruvate dehydrogenase kinase, Pck1: phosphoenolpyruvate carboxykinase 1, Sdh1: succinate dehydrogenase 1, Ucp2: mitochondrial uncoupling protein 2, Pparα1: peroxisome proliferator-activated receptor alpha 1, Acox1: peroxisomal acyl-coenzyme A oxidase 1, Cpt1α: carnitine palmitoyltransferase 1A, Ampk, AMP-activated protein kinase, Acc: acetyl-CoA carboxylase, Sirt1: Sirtuin 1, Pgc1*α*: peroxisome proliferator-activated receptor gamma coactivator 1-α, Nrf1: nuclear respiratory factor 1, Tfam: mitochondrial transcription factor A. F: forward; R: reverse.

Table S2. The relative density of jejunal mucosal metabolites of weanling piglets [1, 2].

Items	Diet treatments			Days post-weaning		*SEM*		*P*-value		
	Control	T 1	T 2	5	21	Diet	Day	Diet	Day	Diet × Day
AKG	1.00	1.22	1.09	1.21	0.99	0.03	0.02	<0.001	<0.001	<0.001
Oxaloacetate	1.00	1.08	1.15	0.96	1.20	0.07	0.06	0.307	0.005	0.047
Succinate	1.00[b]	1.49[a]	1.33[a]	1.31	1.24	0.10	0.08	0.005	0.572	0.091
Phosphoenolpyruvate	1.00[a]	0.55[b]	0.40[b]	0.68	0.62	0.11	0.09	0.001	0.575	0.299
Malate	1.00	0.99	0.97	0.97	1.01	0.02	0.01	0.353	0.053	0.367
Lactate	1.00	1.00	1.04	0.95	1.08	0.04	0.04	0.720	0.016	0.199
Aconitate	1.00	2.40	1.73	1.01	2.41	0.9	0.74	0.556	0.190	0.544
GMP	1.00	1.05	1.23	1.39	0.79	0.18	0.14	0.633	0.006	0.044
NAD^+	1.00	0.85	0.82	1.02	0.76	0.1	0.08	0.388	0.028	0.279
NADH	1.00	1.53	1.97	2.00	1.00	0.45	0.37	0.326	0.062	0.100
NADPH	1.00	0.71	1.45	1.09	1.02	0.06	0.05	<0.001	0.324	<0.001
$NADP^+$	1.00	1.03	1.03	0.88	1.15	0.12	0.10	0.981	0.059	0.295
ADP	1.00	1.27	0.94	1.09	1.05	0.11	0.09	0.080	0.770	0.970
AMP	1.00	1.09	1.54	1.46	0.96	0.11	0.09	0.003	<0.001	<0.001
Cyclic-AMP	1.00	1.03	1.01	1.05	0.98	0.05	0.04	0.897	0.159	0.546
Isocitrate	1.00	1.34	1.33	1.45	1.00	0.08	0.07	0.011	<0.001	0.011
Citrate	1.00	1.13	1.07	1.03	1.10	0.16	0.13	0.831	0.714	0.508
Pyruvate	1.00	1.20	1.17	1.24	1.01	0.06	0.05	0.071	0.003	0.046
Fumarate	1.00	1.14	1.08	1.02	1.13	0.05	0.04	0.122	0.062	0.183
ATP	1.00	1.00	1.08	1.12	0.94	0.10	0.10	0.809	0.133	0.157
GDP	1.00	0.86	0.99	0.98	0.92	0.07	0.06	0.325	0.461	0.436
GTP	1.00	0.79	0.85	0.96	0.81	0.11	0.09	0.416	0.259	0.640
TPP	1.00	0.96	0.84	0.95	0.92	0.08	0.07	0.362	0.752	0.847
FMN	1.00	1.06	1.06	1.01	1.07	0.07	0.05	0.779	0.447	0.702
F-1,6-P	1.00	3.06	1.05	2.48	0.93	0.60	0.49	0.032	0.032	0.029
F-6-P	1.00	1.02	1.07	0.85	1.21	0.10	0.08	0.870	0.004	0.017
3-phosphoglycerate	1.00	0.87	0.69	0.83	0.87	0.07	0.06	0.013	0.605	0.001
G-6-P	1.00	0.99	1.11	0.87	1.19	0.13	0.11	0.749	0.041	0.009
DHAP	1.00	0.81	1.53	1.36	0.86	0.09	0.08	<0.001	<0.001	<0.001

[1] Control, basal diets containing 1.59 % L-alanine (iso-nitrogen); T1, basal diets containing 1 % glutamine, 0.5 % glutamate, 0.1 % aspartate; T2, energy deficiency diets containing 1 % glutamine, 0.5 % glutamate, 0.1 % aspartate. G-6-P: glucose-6-phosphate, F-6-P: fructose 6 phosphate, F-1,6-P: fructose 1,6 phosphate, DHAP: dihydroxyacetone phosphate, AKG: α-ketoglutarate, ATP: adenosine 5 triphosphate, ADP: adenosine 5 diphosphate, AMP: adenosine 5 monophosphate, GTP: guanosine 5 triphosphate, GDP: guanosine 5 diphosphate, GMP: guanosine 5 monophosphate, NAD^+: nicotinamide adenine dinucleotide$^+$, NADH: nicotinamide adenine dinucleotide hydrogen, $NADP^+$: nicotinamide adenine dinucleotide phosphate, NADPH: nicotinamide adenine dinucleotide phosphate hydrogen, TPP: thiamine pyrophosphate, FMN: flavin mononucleotide.

[2] The results were presented as main effects, and the differences among treatments or days were evaluated using Tukey HSD test. [a-b] Values with different letters within the same row are different ($P < 0.05$); n = 6.

Table S3. Relative expressions of gene involved in the energy metabolism[1,2].

Items	Diet treatments			Days post-weaning		*SEM*		*P*-value		
	Control	T 1	T 2	5	21	Diet	Day	Diet	Day	Diet × Day
Jejunal mucosa										
Pdk4	0.99	1.31	0.79	0.95	1.11	0.15	0.13	0.089	0.402	0.009
Pck1	1.00	0.76	0.97	0.77	1.05	0.28	0.24	0.822	0.399	0.038
Sdh1	1.09	2.03	2.01	0.88	2.54	0.33	0.27	0.085	<0.001	0.007
Ucp2	1.00	1.02	1.92	1.54	1.08	0.54	0.43	0.393	0.466	0.407
Pparα1	0.91	1.25	1.91	1.95	0.76	0.68	0.56	0.571	0.147	0.430
Acox1	0.99[b]	1.43[ab]	2.82[a]	2.43	1.07	0.64	0.53	0.012	0.078	0.127
Cpt1α	1.01	1.04	1.15	1.17	0.97	0.29	0.24	0.934	0.569	0.322
Ileal mucosa										
Pdk4	1.00	0.80	1.11	1.07	0.88	0.26	0.21	0.684	0.524	0.807
Pck1	1.01	0.62	0.20	0.67	0.54	0.26	0.21	0.098	0.659	0.748
Sdh1	1.00	0.94	1.36	0.92	1.28	0.19	0.15	0.251	0.115	0.451
Ucp2	1.00[b]	3.23[a]	1.68[ab]	2.64	1.30	0.31	0.25	<0.001	0.001	0.052
Pparα1	0.99	1.02	1.25	1.07	1.10	0.21	0.17	0.640	0.928	0.98
Acox1	1.00	1.30	1.38	1.14	1.31	0.21	0.17	0.415	0.502	0.889
Cpt1α	1.00[b]	0.81[c]	1.39[a]	1.13	1.01	0.05	0.04	<0.001	0.069	0.063

[1] Control, basal diets containing 1.59 % L-alanine (iso-nitrogen); T1, basal diets containing 1 % glutamine, 0.5 % glutamate, 0.1 % aspartate; T2, energy deficiency diets containing 1 % glutamine, 0.5 % glutamate, 0.1 % aspartate. Pdk4: pyruvate dehydrogenase kinase, Pck1: phosphoenolpyruvate carboxykinase 1, Sdh1: succinate dehydrogenase 1, Ucp2: mitochondrial uncoupling protein 2, Pparα1: peroxisome proliferator-activated receptor alpha 1, Acox1: peroxisomal acyl-coenzyme A oxidase 1, Cpt1α: carnitine palmitoyltransferase 1A, ACC: acetyl-CoA carboxylase, SirT1: Sirtuin 1, PGC1*α*: peroxisome proliferator-activated receptor gamma coactivator 1-α, Nrf1: nuclear respiratory factor 1, TFAM: mitochondrial transcription factor A.

[2] The results were presented as main effects, and the differences among treatments or days were evaluated using Tukey HSD test. [a-b] Values with different letters within the same row are different ($P < 0.05$); n = 6.

Table S4. Relative gene expressions of AMPK signaling pathway of jejunal and ileal mucosa of weanling piglets [1,2].

Items	Diet treatments			Days post-weaning		*SEM*		*P*-value		
	Control	T 1	T 2	5	21	Diet	Day	Diet	Day	Diet × Day
Jejunal mucosa										
Ampk	1.00	0.87	1.05	0.64	1.39	0.30	0.31	0.893	0.048	0.364
Acc	1.00	0.99	1.01	0.58	1.41	0.32	0.26	0.999	0.032	0.281
Sirt1	1.04	0.99	1.59	1.45	0.96	0.39	0.32	0.493	0.282	0.449
Pgc1α	0.99^{a}	0.37^{b}	0.86^{a}	0.55	0.89	0.14	0.11	0.013	0.038	0.105
Tfam	1.00^{a}	0.49^{b}	0.78^{a}	0.69	0.83	0.09	0.08	0.003	0.195	0.327
Nrf1	1.00^{a}	0.52^{b}	0.70^{a}	0.65	0.84	0.07	0.05	<0.001	0.016	0.189
Ileal mucosa										
Ampk	1.00	0.96	1.09	0.87	1.40	0.21	0.15	0.295	0.028	0.194
Acc	1.00	0.93	1.18	0.76	1.32	0.15	0.12	0.476	0.003	0.088
Sirt1	1.00^{a}	0.64^{b}	0.86^{a}	0.95	0.72	0.06	0.05	0.001	0.003	0.081
Pgc1α	1.00^{a}	0.33^{b}	0.74^{a}	0.85	0.53	0.13	0.11	0.004	0.042	0.067
Tfam	1.00^{ab}	0.86^{b}	1.22^{a}	1.04	1.01	0.08	0.07	0.017	0.745	0.854
Nrf1	0.99	1.02	1.12	1.06	1.03	0.09	0.07	0.548	0.763	0.617

[1] Control, basal diets containing 1.59% L-alanine (iso-nitrogen); T1, basal diets containing 1% glutamine, 0.5% glutamate, 0.1% aspartate; T2, energy deficiency diets containing 1% glutamine, 0.5% glutamate, 0.1% aspartate. AMPK: AMP-activated protein kinase, ACC: acetyl-CoA carboxylase, SirT1: Sirtuin 1, PGC1*α*: peroxisome proliferator-activated receptor gamma coactivator 1-α, Nrf1: nuclear respiratory factor 1, TFAM: mitochondrial transcription factor A.

[2] The results were presented as main effects, and the differences among treatments or days were evaluated using Tukey HSD test. [a-b] Values with different letters within the same row are different ($P < 0.05$); n = 6.

Table S5. Relative protein abundances of AMPK signaling pathway of jejunal and ileal mucosa of weanling piglets [1,2].

Items	Diet treatments			Days post-weaning		*SEM*		*P*-value		
	Control	T 1	T 2	5	21	Diet	Day	Diet	Day	Diet × Day
Jejunal mucosa										
AMPK	0.99	1.09	1.15	1.31	0.85	0.05	0.04	0.084	<0.001	<0.001
P-AMPK	0.99	0.67	0.87	0.95	0.74	0.05	0.04	0.001	0.002	<0.001
ACC	0.99	0.67	0.86	1.11	0.57	0.05	0.04	0.001	<0.001	<0.001
P-ACC	0.99	0.80	1.25	1.38	0.65	0.09	0.07	0.013	<0.001	0.001
SirT1	0.99	0.67	1.08	1.21	0.63	0.05	0.04	<0.001	<0.001	<0.001
PGC1a	0.99	0.88	1.12	1.36	0.64	0.09	0.07	0.172	<0.001	<0.001
Ileal mucosa										
AMPK	0.99	0.81	0.55	0.99	0.58	0.05	0.04	<0.001	<0.001	<0.001
P-AMPK	0.99	0.48	0.90	0.88	0.70	0.06	0.05	<0.001	0.030	0.040
ACC	0.99^{b}	1.34^{a}	1.19^{ab}	1.25	1.09	0.05	0.04	0.002	0.023	0.222
P-ACC	0.99	0.64	0.72	1.01	0.56	0.07	0.05	0.007	<0.001	0.004
SirT1	0.99	0.52	1.05	1.08	0.63	0.05	0.04	<0.001	<0.001	<0.001
PGC1a	0.99	0.45	1.04	1.07	0.59	0.05	0.04	<0.001	<0.001	<0.001

[1] Control, basal diets containing 1.59% L-alanine (iso-nitrogen); T1, basal diets containing 1% glutamine, 0.5% glutamate, 0.1% aspartate; T2, energy deficiency diets containing 1% glutamine, 0.5% glutamate, 0.1% aspartate. AMPK: AMP-activated protein kinase, ACC: acetyl-CoA carboxylase, SirT1: Sirtuin 1, PGC1*α*: peroxisome proliferator-activated receptor gamma coactivator 1-α, Nrf1: nuclear respiratory factor 1, TFAM: mitochondrial transcription factor A.

[2] The results were presented as main effects, and the differences among treatments or days were evaluated using Tukey HSD test. [a-b] Values with different letters within the same row are different ($P < 0.05$); n = 6.

Figures

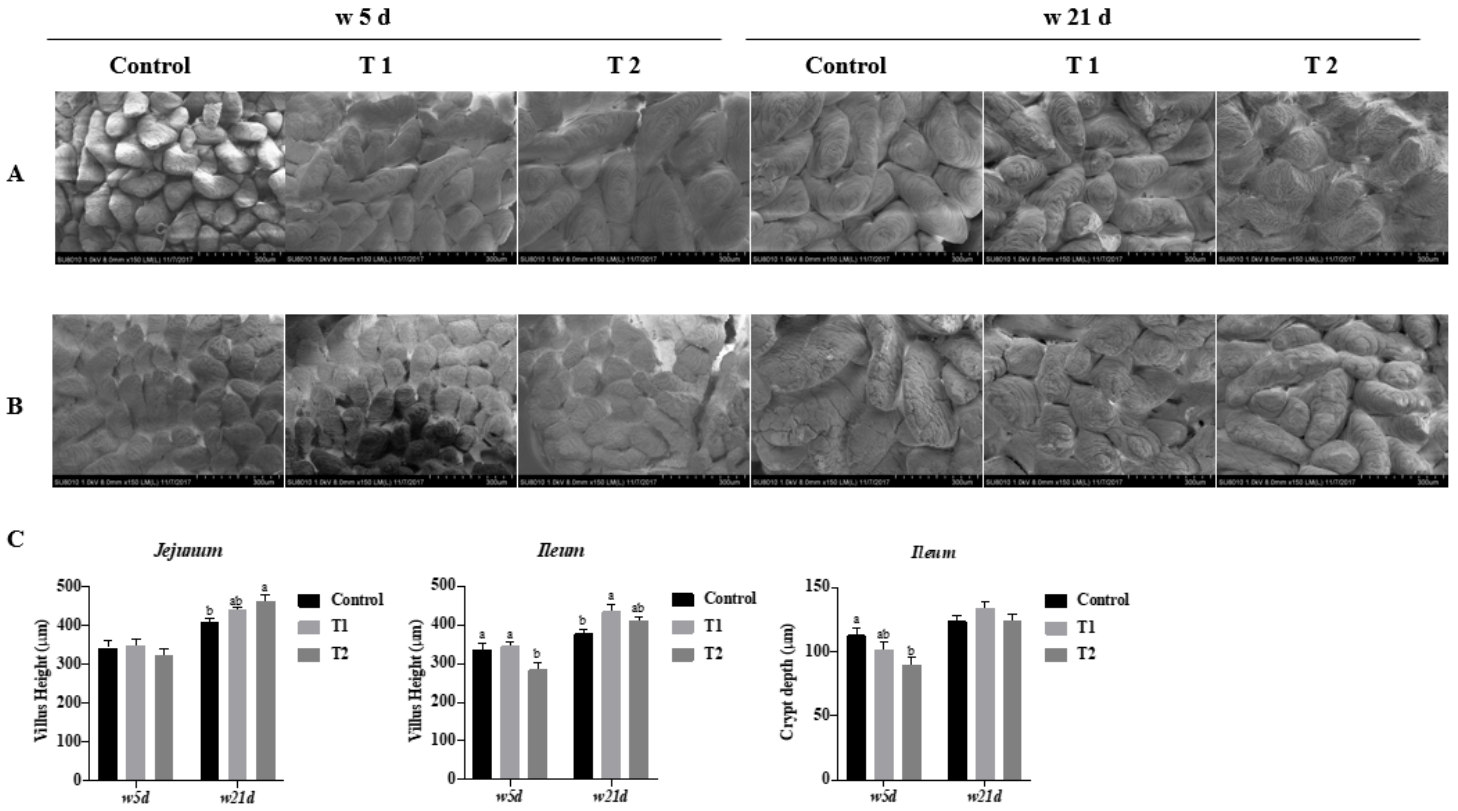

Figure 1

Small intestinal morphology of weaning piglets. The representative images of scanning electron microscopy in the jejunum (A) and ileum (B) of piglets on days 5 (w 5 d), 21 (w 21 d) post-weaning at 21 d of age (magnification × 150, scale bar = 300 μm) (n = 6). (C) Histogram of the jejunal and ileal villus height and crypt depth detected by hematoxylin-eosin staining. Data are presented as the simple main effect analysis and expressed as means ± SEM, n=6. a-b Values with different lowercase letters are different ($P < 0.05$). Control: basal diets containing 1.59 % L-alanine (iso-nitrogen); T 1: basal diet containing 1 % L-glutamine, 0.5 % L-glutamate, and 0.1 % L-aspartate; T 2: energy deficiency diet containing 1 % L-glutamine, 0.5 % L-glutamate, and 0.1 % L-aspartate.

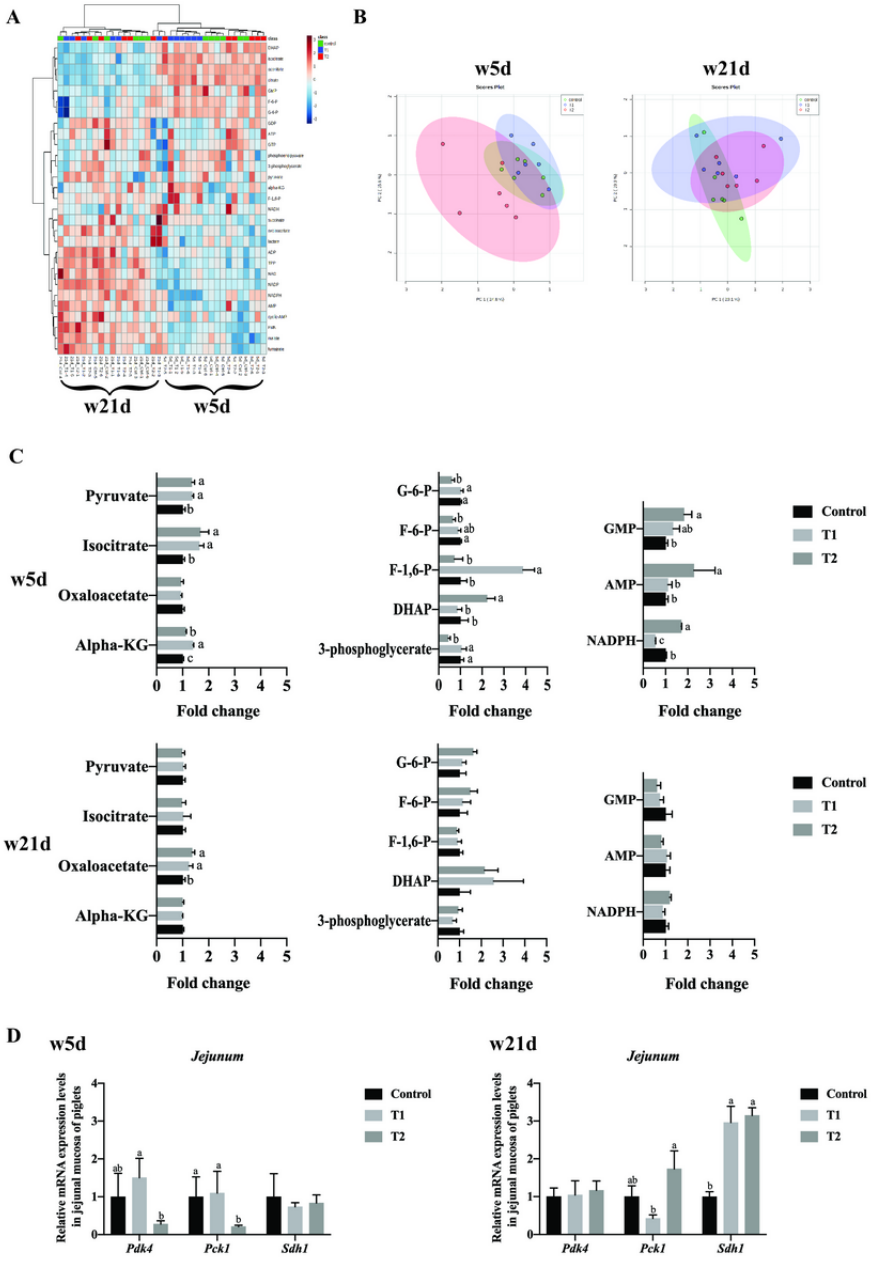

Figure 2

Regulation of jejunal mucosal energy metabolism by glutamine, glutamate, and aspartate in weaning piglets. (A) Heat map of the metabolites of jejunal mucosa in weaning piglets on days 5 (w 5 d), 21 (w 21 d) post-weaning. (B) Principal components analysis of jejunal mucosal metabolites in weaning piglets on days 5 (w 5 d), 21 (w 21 d) post-weaning. (C) Representative variation of energy metabolites of jejunal mucosa in weaning piglets on days 5 (w 5 d), 21 (w 21 d) post-weaning. (D) Relative mRNA expressions of pyruvate dehydrogenase kinase (Pdk4), phosphoenolpyruvate carboxykinase 1 (Pck1), and succinate dehydrogenase 1 (Sdh1) in jejunal mucosa of weaning piglets on days 5 (w 5 d), 21 (w 21 d) post-weaning. Data are presented as the simple main effect analysis and expressed as means ± SEM, n=6. a-c Values with different lowercase letters are different ($P < 0.05$). Control: basal diets containing 1.59 % L-alanine (iso-nitrogen); T 1: basal diet containing 1 % L-glutamine, 0.5 % L-glutamate, and 0.1 % L-aspartate; T 2: energy deficiency diet containing 1 % L-glutamine, 0.5 % L-glutamate, and 0.1 % L-aspartate.

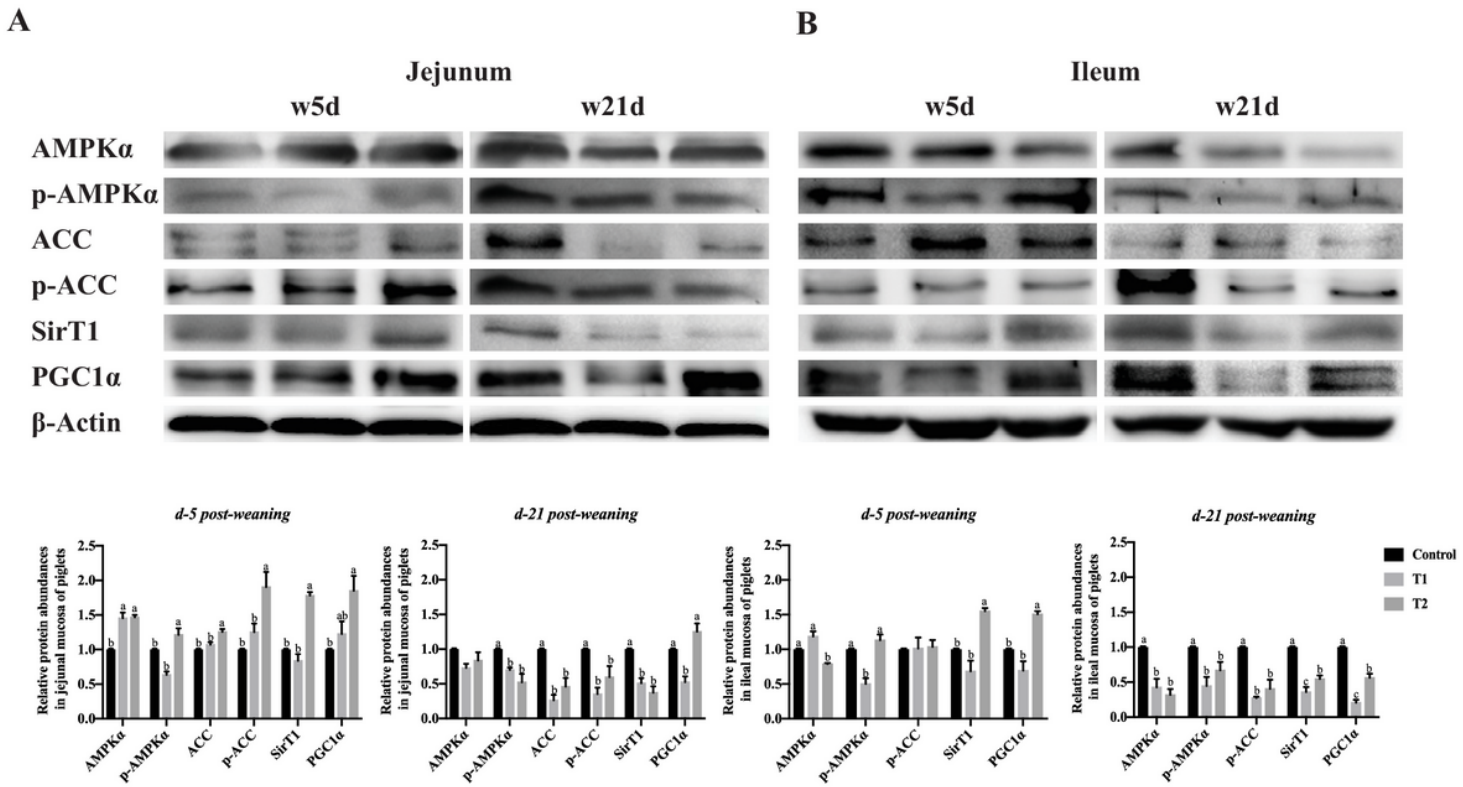

Figure 3

Relative protein abundances of AMP-activated protein kinase (AMPK) signaling pathway in jejunal (A) and ileal (B) mucosa of weaning piglets on days 5 (w 5 d), 21 (w 21 d) post-weaning. Data are presented as the simple main effect analysis and expressed as means ± SEM, n=6. a-c Values with different lowercase letters are different ($P < 0.05$). Control: basal diets containing 1.59 % L-alanine (iso-nitrogen); T 1: basal diet containing 1 % L-glutamine, 0.5 % L-glutamate, and 0.1 % L-aspartate; T 2: energy deficiency diet containing 1 % L-glutamine, 0.5 % L-glutamate, and 0.1 % L-aspartate. ACC: acetyl-CoA carboxylase, SirT1: Sirtuin 1, PGC1α: peroxisome proliferator-activated receptor gamma coactivator 1-α.

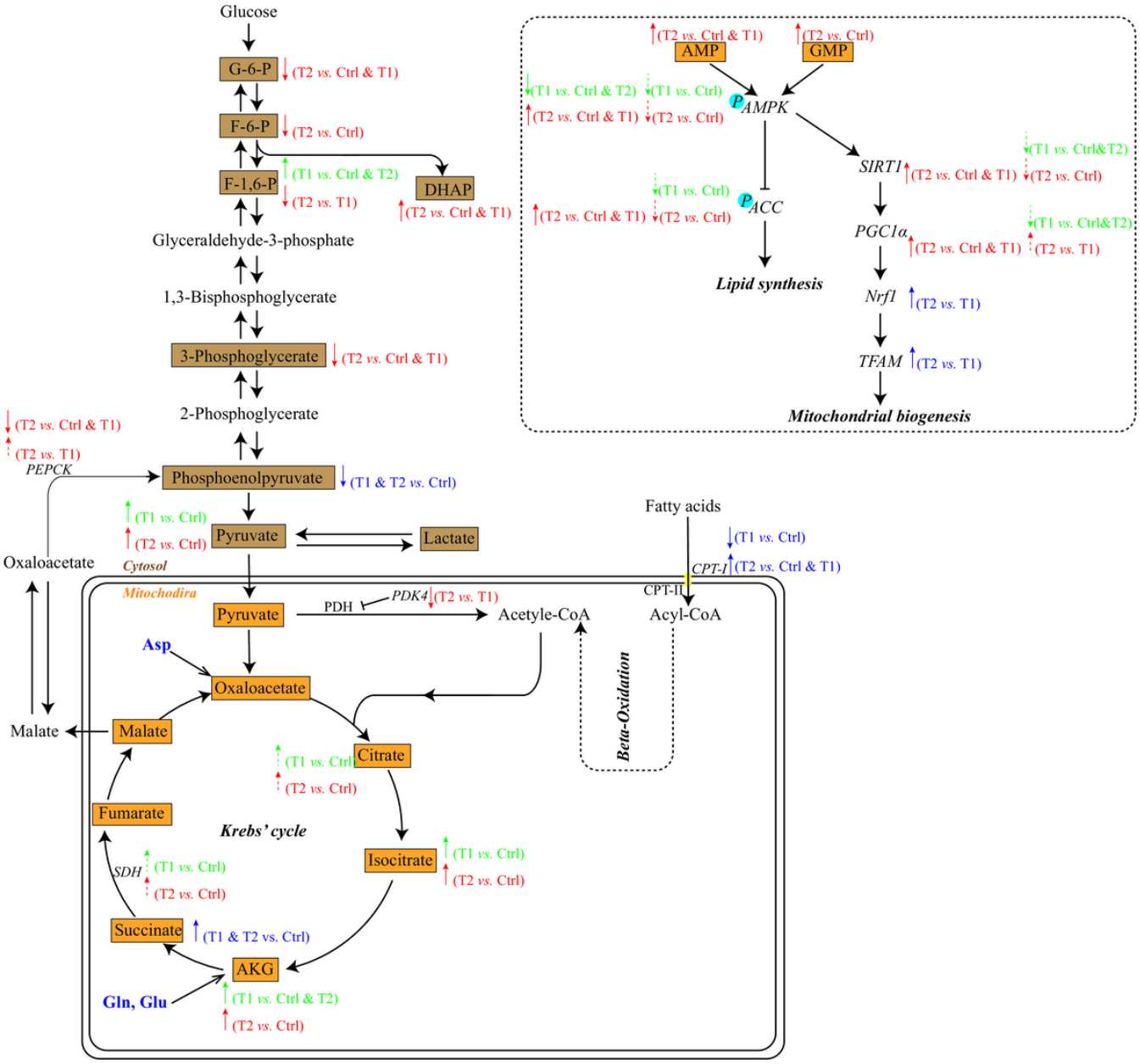

Figure 4

Schematic representation of main changes of the energy metabolites and enzymes involved in the glycolysis, Krebs' cycle, and AMP-activated protein kinase (AMPK) (dashed rectangular) pathways identified in jejunal mucosa of weaning piglets. The filled rectangular and italics represent the metabolite and enzyme are measured in current study. The solid and dashed arrows (↑, increase; ↓, decrease) in green color represent the significant changes in the T1 treatment on d 5 and 21 post-weaning, respectively. The solid and dashed arrows (↑, increase; ↓, decrease) in red color represent the significant changes in the T2 treatment on d 5 and 21 post-weaning, respectively. The solid arrows (↑, increase; ↓, decrease) in blue color represent the main effect of three treatments (Control, T1, T2) when there is no significant interaction between treatment and days. Control: basal diets containing 1.59 % L-alanine (iso-nitrogen); T 1: basal diet containing 1 % L-glutamine, 0.5 % L-glutamate, and 0.1 % L-aspartate; T 2: energy deficiency diet containing 1 % L-glutamine, 0.5 % L-glutamate, and 0.1 % L-aspartate. G-6-P: glucose-6-phosphate, F-6-P: fructose 6 phosphate, F-1,6-P: fructose 1,6 phosphate, DHAP: dihydroxyacetone phosphate, AKG: α-ketoglutarate, AMP: adenosine 5 monophosphate, GMP: guanosine 5 monophosphate, PDH: pyruvate dehydrogenase, PDK4: pyruvate dehydrogenase kinase, SDH: succinate dehydrogenase 1, PEPCK: phosphoenolpyruvate carboxykinase, CPTI: carnitine palmitoyltransferase 1, CPTII: carnitine palmitoyltransferase 2, ACC: acetyl-CoA carboxylase, SirT1: Sirtuin 1, PGC1α:

peroxisome proliferator-activated receptor gamma coactivator 1-α, Nrf1: nuclear respiratory factor 1, TFAM: mitochondrial transcription factor A.