Original Research Article

Glutamine, glutamate, and aspartate differently modulate energy homeostasis of small intestine under normal or low energy status in piglets

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ABSTRACT

Weaning stress may cause reduced energy intake for maintenance of mucosal structure. Gln, Glu, and Asp are major energy sources for the small intestine. This study investigated whether Gln, Glu, and Asp improve the intestinal morphology via regulating the energy metabolism in weaning piglets. A total of 198 weaned piglets were assigned to 3 treatments: Control (Basal diet + 1.59% L-Ala); 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 21 post-weaning. T1 enhanced growth performance. T1 and T2 treatments improved small intestinal morphology by increasing villus height, goblet cell number and decreasing crypt depth. Days post-weaning affected the efficacy of T2, but not T1, on energy metabolism. At normal energy supplementation, Gln, Glu, and Asp restored small intestinal energy homeostasis via replenishing the Krebs' cycle and down-regulating the AMPK (adenosine monophosphate activated protein kinase) pathway. As these are not sufficient to maintain the intestinal energy-balance of piglets fed with a low energy diet on d 5 post-weaning, the AMPK, glycolysis, beta-oxidation, and mitochondrial biogenesis are activated to meet the high energy demand of enterocytes. These data indicated that Gln, Glu, and Asp could restore the energy homeostasis of intestinal mucosa of weaning piglets under normal energy fed. Low energy feeding may increase the susceptibility of piglets to stress, which may decrease the efficacy of Gln, Glu, and Asp on the restoration of energy balance. These findings provide new information on nutritional intervention for insufficient energy intake in weaning piglets.

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1. Introduction

At weaning, young pigs are subjected to removal from the sow and littermates and are transported to a new environment, and their diet is abruptly changed from sow's milk to a solid diet, which causes low voluntary food intake and associated poor intestinal barrier structure and function (Pluske et al., 1997; Xiao et al., 2014). Undoubtedly, the weaning piglets are not eating enough food and their intestinal mucosa is failing to digest and absorb sufficient nutrients to cover their energy requirement for maintenance (Dreau et al., 1994), which are major limitations to improving the growth of piglets after weaning. Additionally, the porcine

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gastrointestinal (GI) tract has a more similar morphology and function to the human GI tract than other non-primate species (Sciascia et al., 2016). During the weaning process, the high energy requirement of human offspring is supported by the provision of high energy-dense and easily digestive foods to reduce poor offspring outcomes (Humphrey, 2010). Therefore, improving the energy intake from exogenous nutrition is rationally served as a promising approach to protect both young livestock animals and human offspring from weaning stress.

The intestine of a piglet has a notably high energy demand due to the rapid renewal of epithelium within a few days (Madej et al., 2002; Wiese et al., 2003). Hence, the epithelial cells of the GI tract require intense anabolic metabolism. It has been demonstrated that the GI tract represents approximately 5% of body weight, whereas it is responsible for about 20% of whole body O_2 consumption (Virbasius and Scarpulla, 1994). Glucose and lipids are major sources for the supply and storage of energy in cells (Garcia and Shaw, 2017). AMP-activated protein kinase (AMPK) is the master regulator of energy metabolism (Virbasius and Scarpulla, 1994). AMPK increases adenosine triphosphate (ATP) levels by promoting glucose and lipids breakdown and inhibiting their synthesis and storage (Garcia and Shaw, 2017). Glycolysis, Krebs' cycle, and fatty acids beta-oxidation are the main catabolic processes for glucose and lipids (Lodish and Zipursky, 2001), respectively. Although all animals obtain their biological energy from the cell-specific oxidation of fatty acids, glucose, and amino acids in diets (Jobgen et al., 2006), for the gastrointestinal tract, Gln, Glu, and Asp are the main energy sources to maintain gut integrity and function (Wu, 2013; Hou et al., 2015). Meanwhile, numerous studies have reported that Gln, Glu, and Asp have profound impacts on intestinal nutrition and health. Gln plays multiple roles in regulating intestinal protein turnover (Wu et al., 2014), gene expression (Zhu et al., 2015), cell proliferation (Kim et al., 2013) and immune function (Ren et al., 2014). Asp could improve growth performance of weaning piglets (Li et al., 2018), and attenuate the intestinal injury induced by *Escherichia coli* lipopolysaccharide (LPS) (Wang et al., 2017). Nevertheless, young piglets could not synthesize sufficient Asp, Glu and Gln, and a typical corn- and soybean meal-based diet also cannot provide sufficient amounts of Asp, Glu, and Gln for protein synthesis (Wu et al., 2010). It has been demonstrated that most dietary Gln and almost all Glu and Asp cannot enter into the portal circulation but is catabolized to CO_2 in the small intestine. Thus, we assumed that dietary supplementation with Gln, Glu and Asp may restore the intestinal energy homeostasis that is disturbed by weaning stress through regulating mucosal energy metabolism in piglets. However, the insight mechanism still needs to be further illustrated. In addition, to our knowledge, this is the first study that combines Gln, Glu, and Asp, the major energy sources of the small intestine, to investigate the synergistic regulation of intestinal energy homeostasis.

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

2. Materials and 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).

2.1. 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: 1) Control (Basal diet + 1.59% L-Ala; iso-nitrogenous control); 2) T1 (Basal diet + 1% L-Gln + 0.5% L-Glu + 0.1% L-Asp); 3) 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. Previous study has demonstrated that dietary supplementation with 0.07% Asp and 0.5% Glu, respectively, could improve the growth performance and circulating amino acid pool of piglets (Li et al., 2018). 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.

Table 1
Ingredients and composition (as-fed basis) of diets (%).[1]

Item	Control diet	T1 diet	T2 diet
Ingredients			
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[4]			
Digestible energy, kcal/kg	3,445.60	3,444.56	3,227.00
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] T1 = Basal diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp; T2 = energy deficiency diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp; the premix did not contain additional copper, zinc, antibiotics, or probiotics.

[2] Mineral premix provided for 1 kg of completed diet: Zn (ZnO), 50 mg; Cu (CuSO$_4$), 20 mg; Mn (MnO), 55 mg; Fe (FeSO$_4$), 100 mg; I (KI), 1 mg; Co (CoSO$_4$), 2 mg; Se (Na$_2$SeO$_3$), 0.3 mg.

[3] Vitamin premix provided for 1 kg completed diet: vitamin A, 8,255 IU; vitamin D$_3$, 2,000 IU; vitamin E, 40 IU; vitamin B$_1$, 2 mg; vitamin B$_2$, 4 mg; pantothenic acid, 15 mg; vitamin B$_6$, 10 mg; vitamin B$_{12}$, 0.05 mg; nicotinic acid, 30 mg; folic acid, 2 mg; vitamin K$_3$, 1.5 mg; biotin, 0.2 mg; choline chloride, 800 mg; and vitamin C, 100 mg.

[4] Calculated values.

One piglet close to the average body weight from each pen was slaughtered on d 5 and 21 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.

2.2. 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; 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 5 randomly selected fields, respectively (Montagne et al., 2007).

Segments of the jejunum and ileum at 150-fold magnification were also designated for analysis by scanning electron microscopy as described by German (2009) and Liu et al. (2013). Briefly, tissue segments were fixed with 2.5% glutaraldehyde for 2 h at 4 °C, and rinsed 10 min × 3 times in phosphate buffered saline (PBS) at 4 °C. The tissues were then fixed in 1% osmium tetroxide for 12 h at 4 °C, and rinsed 10 min × 3 times 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 the microvillus were observed and described.

2.3. 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 double-distilled H_2O (ddH_2O), and mixed with 800 μL methanol−acetonitrile (1:1, vol:vol) and sonicated in an 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, and resuspended in 100 μL methanol/ddH_2O (1:1, vol:vol) for LC-MS/MS analysis. An aliquot from each sample was pooled to create quality control 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 × 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 2 solutions: (A) 15 mmol/L 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 ψ; ion source gas 2, 45 ψ; curtain gas, 30 ψ; ionSapary voltage floating, 4,500 V.

2.4. Real-time quantitative reverse transcriptase PCR

Expressions of pyruvate dehydrogenase kinase 4 (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 (Wang et al., 2015b). 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 (Appendix Table 1). 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.

2.5. Western blotting

Jejunal and ileal mucosa samples were homogenized, and protein concentrations were measured using the bicinchoninic acid assay method with bovine serum albumin (BSA) as standard (Catalogue #P0010, 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 sample buffer and heated in boiling water for 5 min and cooled down for Western blot analysis (Wang et al., 2016a). The following first antibodies were used for protein quantification: AMPKα (1:1,000; Catalogue # 2532, Cell Signaling Technology, USA), phosphorylated AMPKα (p-AMPK (Thr175); 1:1,000; Catalogue # 2535, Cell Signaling Technology, USA), ACC (1:1,000; Catalogue # 3662, Cell Signaling Technology, US), phosphorylated ACC (p-ACC (Ser79); 1:1,000; Catalogue # 3661, Cell Signaling Technology, USA), SirT1 (1:1,000; Catalogue # 9475, Cell Signaling Technology, USA), PGC1α (1:1,000; Catalogue # ab191838, Abcam, UK) and β-actin (1:1,000; Catalogue # 4970, Cell Signaling Technology, USA) as well as secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit IgG; 1:5,000; Catalogue # ZB2301, Boster Biological Technology, Wuhan, China). The images were detected by chemiluminescence (Catalogue # WBKLS0100, 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.

2.6. 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 are 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 Appendix Tables 3—5). 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 Figs. 1—3). The developmental
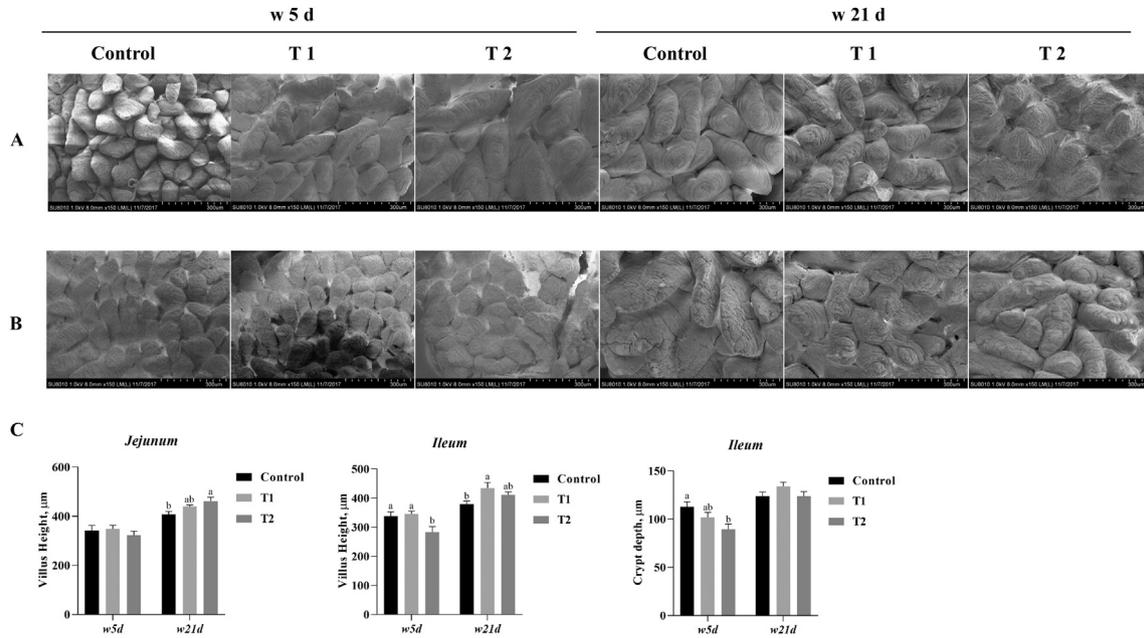
Fig. 1. Small intestinal morphology of weaning piglets. The representative images of scanning electron microscopy in the jejunum (A) and ileum (B) of piglets on d 5 and 21 post-weaning at 21 d of age (magnification 150×, scale bar = 300 μm) ($n = 6$). (C) Histogram of the jejunal and ileal villus height 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-Ala (iso-nitrogen); T1 = basal diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp; T2 = energy deficiency diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp.

changes of the intestine were not the main outcome, so the differences between d 5 and 21 post-weaning across 3 levels of treatments are not shown. Differences were declared as significant at $P < 0.05$.

3. Results

3.1. Growth performance

Initial 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) are shown 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 the control group ($P < 0.05$). However, the digestible energy intakes of weaning piglets were not significantly different, neither on d 5 ($P = 0.190$) nor on d 21 ($P = 0.709$) post-weaning. In addition, we did not record the diarrhea rate in the current study.

3.2. Jejunal and ileal morphology

In Fig. 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 the control, T1, and T2 groups did not show as significantly different on d 5 post-weaning, but there was an increase in villi density and villi-fold in the T1 and T2 groups on d 21 post-weaning. In the ileum, increased villi densities were observed in the control and T1 groups in comparison to the T2 group on d 5 post-weaning, whereas villi in

the T1 and T2 groups were denser and stouter than villi in the control group on d 21 post-weaning.

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

3.3. Metabolomic profiling of jejunal mucosa of weaning piglets

The jejunal mucosa energy metabolite fingerprints are presented in Fig. 2A and B. Piglets on d 5 and 21 post-weaning could be readily differentiated by the heat map of their jejunal mucosa energy metabolites (Fig. 2A). Piglets on d 5 post-weaning showed a greater abundance of dihydroxyacetone phosphate (DHAP), iso-citrate, aconitate, citrate, guanosine 5 monophosphate (GMP), fructose 6 phosphate (F-6-P), and glucose-6-phosphate (G-6-P) in the jejunal mucosa, whereas piglets on d 21 post-weaning showed a greater abundance of fumarate, malate, flavin mononucleotide (FMN), cyclic-adenosine 5 monophosphate (c-AMP), AMP, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH),
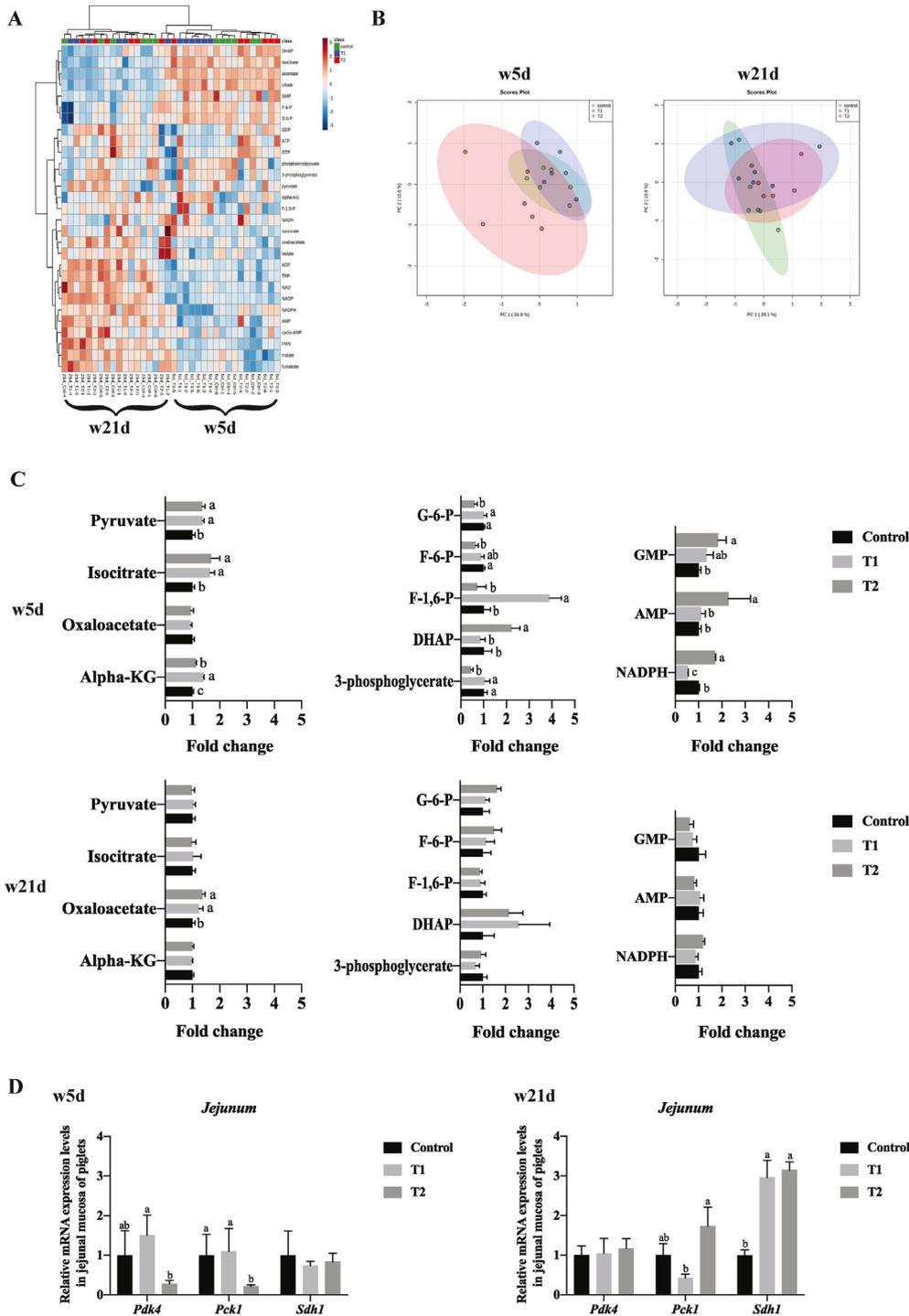
Fig. 2. Regulation of jejunal mucosal energy metabolism by Gln, Glu, and Asp in weaning piglets. (A) Heat map of the metabolites of jejunal mucosa in weaning piglets on d 5 and 21 post-weaning. (B) Principal components analysis of jejunal mucosal metabolites in weaning piglets on d 5 and 21 post-weaning. (C) Representative variation of energy metabolites of jejunal mucosa in weaning piglets on d 5 and 21 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 d 5 and 21 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-Ala (iso-nitrogen); T1 = basal diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp; T2 = energy deficiency diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp.

nicotinamide adenine dinucleotide phosphate ($NADP^+$), nicotinamide adenine dinucleotide (NAD^+), adenosine 5 diphosphate (ADP), and thiamine pyrophosphate (TPP) in the jejunal mucosa. The first two components of a principal component analysis showed that the jejunal mucosal energy metabolites of the control and T1 treatments on d 5 post-weaning were comparable, whereas

T2 treatment on d 5 post-weaning was more dispersive and different from the control and T1 treatments. On d 21 post-weaning, energy metabolites in the 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 are shown in Appendix
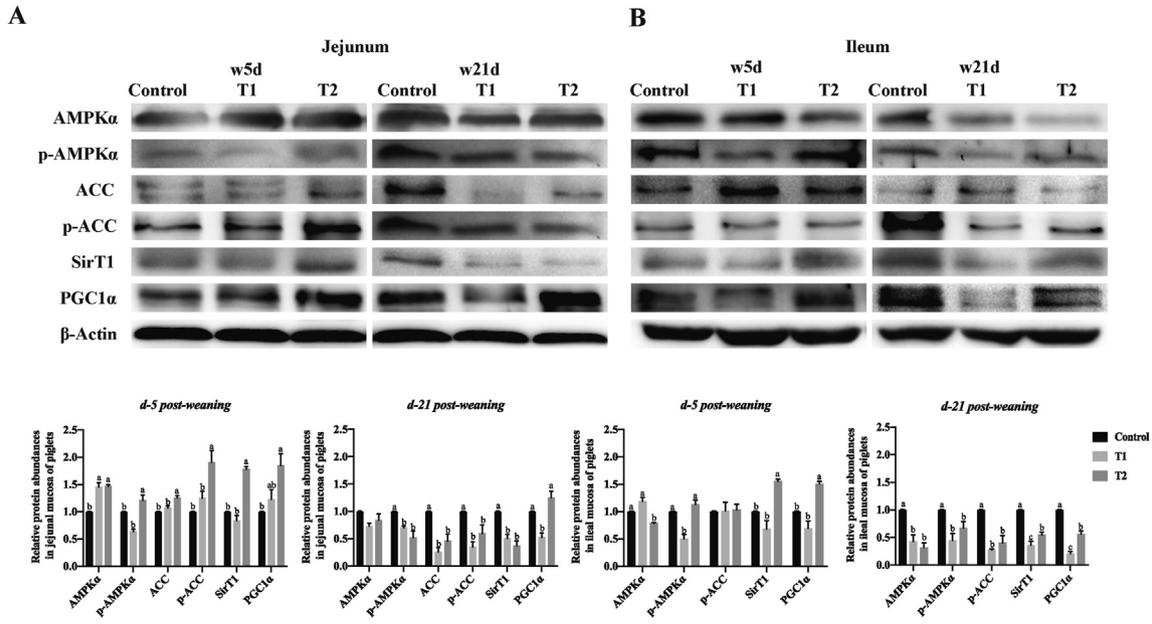
Fig. 3. Relative protein abundances of AMPK pathway in jejunal (A) and ileal (B) mucosa of weaning-piglets. Data are presented as the simple main effect analysis and expressed as means ± SEM, n = 6. [a, b] Values with different lowercase letters are different (P < 0.05). Control = basal diets containing 1.59% L-Ala (iso-nitrogen); T1 = basal diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp; T2 = energy deficiency diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp. AMPK = AMP-activated protein kinase, ACC = acetyl-CoA carboxylase, SirT1 = Sirtuin 1, PGC1α = peroxisome proliferator-activated receptor gamma coactivator 1-α.

Table 2
Growth performance of weaning piglets.

Item	Treatments[1]			SEM	P-value
	Control	T1	T2		
Day 5 post-weaning					
Initial weight, kg	7.09	7.05	7.09	0.070	0.959
BW at d 5 post-weaning, 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, kcal/pig	238.22	307.27	302.91	16.61	0.190
Day 21 post-weaning					
BW at d 21 post-weaning, 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, kcal/pig	1,644.99	1,814.33	1,692.69	83.900	0.709

[a, b] Values with different letters within the same row are different (P < 0.05); n = 6.
[1] T1 = Basal diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp; T2 = energy deficiency diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp; BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; F/G = gain: feed ratio.

Table 2. When significant interactions between treatments and days after weaning were found, differences among the control, T1 and T2 on d 5 and 21 post-weaning are shown in Fig. 2C. In Appendix Table 2, 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 the jejunal mucosa of weaning piglets. Days post-weaning had influences on the diet treatments' efficacy (P interaction < 0.05). As shown in Fig. 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 the 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 the 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.

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

The gene expressions of several key enzymes of energy metabolism are presented in Appendix Table 3 and Fig. 2D. The main effect in Appendix Table 3 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 the

Table 3
Jejunal and ileal morphology of weaning piglets[1].

Item	Diet treatments[2]			Days post-weaning		SEM		P-value		
	Control	T1	T2	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[3]	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[3]	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

[a-b] Values with different letters within the same row are different ($P < 0.05$); [*] means the difference was significant when compared to d 5 post-weaning ($P < 0.05$); $n = 6$.
[1] The results were presented as main effects, and the differences among treatments or days were evaluated using Tukey HSD test.
[2] T1 = Basal diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp; T2 = energy deficiency diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp; VCR = the ratio of villus height to crypt depth.
[3] The numbers of goblet cells and lymphocytes were averaged from 4 fields at 400-fold magnification.

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 ($P_{interaction} < 0.05$; Fig. 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 the 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 the T1 or control groups, respectively.

3.5. 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 the AMPK pathway in the jejunal and ileal mucosa are presented in Appendix Tables 4 and 5, and Fig. 3, respectively. The main effects on AMPK pathway gene expressions in Appendix Table 4 showed that 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 the control and T2 groups, as well as ileal *Tfam* mRNA level compared to the T2 group. The main effects on AMPK pathway protein abundances in Appendix Table 5 showed that 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 influenced the treatments' efficacy on AMPK pathway protein abundances ($P_{interaction} < 0.05$). As shown in Fig. 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 the 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 the control and T1 groups, but increased ($P < 0.05$) jejunal and ileal SirT1 and PGC1α, as well as jejunal ACC and p-ACC protein abundances compared to the 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.

4. Discussion

The mammalian intestine plays a key role in nutrient intake and has a high rate of energy expenditure because the digestion and absorption processes are highly dependent on energy (Van Der Schoor et al., 2002; Wang et al., 2016b). Weaning results in a growth check caused by a period of low feed intake, which also reduces the energy intake of the intestine of piglets (Bruininx et al., 2001; Varley and Wiseman, 2001). Successful adaptation to these changes requires profound morphological and energy metabolic adjustments of the gastrointestinal tract (Marion et al., 2002). Accumulating evidence has shown the beneficial effect of Gln, Glu, and Asp in piglets under physiological and pathological conditions (Li et al., 2018; Chen et al., 2021), as well as the combination protective effect of Glu and Asp on piglets induced by oxidative stress (Duan et al., 2016). The aim of the current study is to use the main energy sources of the small intestine, Gln, Glu, and Asp, to help the faster restitution of the mucosal barrier after stress injury, and to improve the intestinal energy metabolism of piglets with different energy level diets.

The most serious damage in piglets during weaning-stress occurs on d 2 to 5 post-weaning (Wu et al., 2015; Wang et al., 2016b), and is usually associated with a dramatic reduction of feed intake resulting in marked changes in the growth pattern on body weight (Marion et al., 2002). 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 studies, which have shown that Glu and Asp exert positive effects on growth performance of animals in healthy and oxidative-stress conditions (Wu, 2010; Yin et al., 2015; Duan et al., 2016; Chen et al., 2018b). Although the calculated digestive energy intake in the T2 group was comparable with these in the control and T1

groups, the low energy diet did not significantly increase the BW and ADG of weaning piglets. Meanwhile, Glu, as the precursor of gamma-aminobutyric acid, may contribute to the appetite of piglets (Sterndale et al., 2017), which may explain the trend of ADFI of piglets in the T1 and T2 groups to be higher than that in the control group.

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 (van Beers-Schreurs et al., 1998; Hu et al., 2013). Supplementation with Gln, Glu, and Asp in a 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 (Wu and Knabe, 1994; Wu et al., 1995). 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 and 21 post-weaning. The critical roles of these 3 amino acids in the intestine have been reported in many well-designed studies. For example, Gln could increase the villus length of jejunum (He et al., 2016), and Asp could alleviate intestinal barrier damage (Pi et al., 2014; Kang et al., 2015; Duan et al., 2016). The results in our current study indicated that Gln, Glu, and Asp could improve the intestinal barrier structure of weaning piglets fed either a low energy diet or a normal energy diet, which led us to further explore the mechanism of these 3 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 21 post-weaning were not comparable, and the regulation of Gln, Glu, and Asp on energy metabolites varied between d 5 and 21 post-weaning. This may indicate that the energy metabolism in the small intestine changes during the weaning period (Wang et al., 2018), 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 (Wu, 2009, 2013; Yao et al., 2012). After these 3 amino acids are absorbed into enterocytes, they could be metabolized and oxidized via the Krebs' cycle to water and CO_2 yielding ATP (Rezaei et al., 2013). The oxidative steps of Gln and Glu in pig enterocytes are similar. Gln firstly enters the mitochondria in order to be degraded to Glu and ammonia by the mitochondrial glutaminase (Wang et al., 2015a). Glu could be transaminated to produce AKG, which is the key molecule of the Krebs' cycle (Blachier et al., 2009). Asp replenished the Krebs cycle by being converted to oxaloacetate (Rezaei et al., 2013). 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 and 21 post-weaning. Meanwhile, supplementation with Gln, Glu and Asp in either a regular energy diet or a low energy diet enhanced $Sdh1$ mRNA abundance on d 21 post-weaning, whereas a low energy diet supplemented with Gln, Glu, and Asp declined $Pdk4$ mRNA abundance on d 5 post-weaning. SDH1 is part of the Krebs' cycle and oxidizes succinate to fumarate (Rutter et al., 2010). 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 (Connors et al., 2019). PDK4 is a regulator of pyruvate dehydrogenase (PDH), as it could inactivate PDH by phosphorylation (Lundsgaard et al., 2017). PDH represents a cornerstone in cellular energy metabolism, linking glycolysis and the Krebs' cycle and lipid metabolism (Lazzarino et al., 2019). In this study, no PDH activity was detected, whereas the decreased $Pdk4$ gene expression might suggest that the influx of acetyl-CoA from glycolysis into the Krebs' cycle increased in response to the 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 the jejunal mucosa on d 5 post-weaning, whereas the T2 treatment 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, neither on d 5 nor on 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 enterocytes 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 a critical role in energy homeostasis (Houten and Wanders, 2010). UCP2 is involved in the regulation of mitochondrial substrate oxidation, which could be stimulated by fatty acid and glutamine (Hurtaud et al., 2007; Bouillaud et al., 2016). 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 (Criscuolo et al., 2006; Pecqueur et al., 2008). Indeed, a normal energy diet supplementation with Gln, Glu, and Asp raised the $Ucp2$ mRNA abundance but decreased $Cpt1\alpha$ 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 $Cpt1\alpha$ 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 (Schlaepfer and Joshi, 2020), whereas Acox1 is the first enzyme of the fatty acid beta-oxidation in peroxisomes, which catalyzes the desaturation of acyl-CoA to 2-trans-enoyl-CoA (Bouagnon et al., 2019). 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 the pentose cycle, which is an alternative way of using glucose (Werner et al., 2016). 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 (Burgess et al., 2007), may implicate the reduced gluconeogenesis in the T2 group on d 5 post-weaning. The higher AMP and GMP levels activate 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 (Carling, 2019). Thus, it is reasonable to speculate that a low energy 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. Low energy conditions may activate AMPK to phosphorylate downstream substrates, and increase ATP levels by promoting glucose and lipids breakdown, inhibiting their synthesis and storage (Garcia and Shaw, 2017; Chen et al., 2018a). 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,

simultaneously promoting fatty acid oxidation by relieving the suppression of CPT1 (Garcia and Shaw, 2017). Another interesting finding is that the PGC1α gene and protein abundances were enhanced by a low energy diet supplementation with Gln, Glu, and Asp (T2). PGC1α provides a direct link between external physiological stimuli and the regulation of mitochondrial biofunction (Gureev et al., 2019). PGC1α regulates mitochondrial biogenesis via regulation of Nrf1 (Finck and Kelly, 2007), which in turn activates TFAM that is involved in the mDNA replication (Virbasius and Scarpulla, 1994). In the current study, the increased *Nrf1* and *Tfam* gene expression in the 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 but down-regulated the phosphorylated AMPK
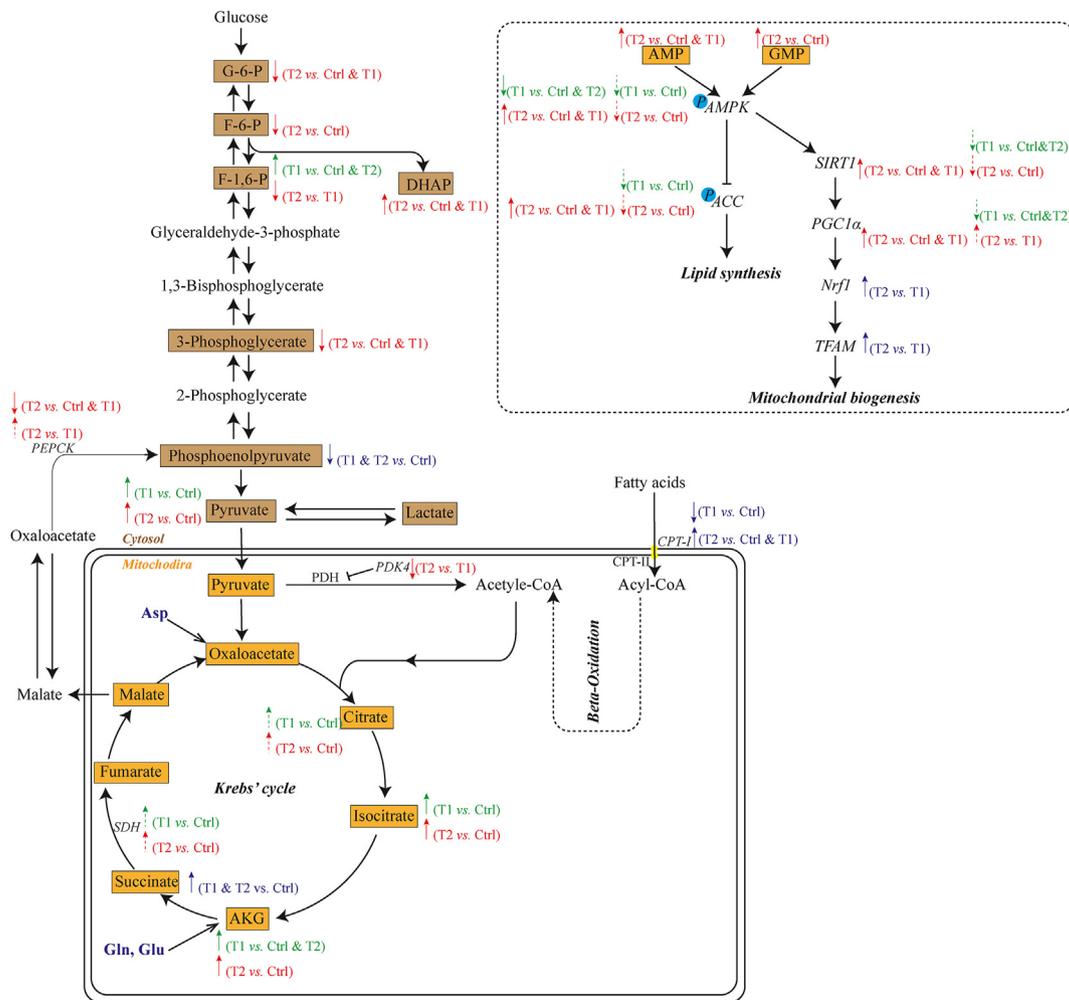
Fig. 4. Schematic representation of main changes of energy metabolites and enzymes in jejunal mucosa of weaning-piglets. The filled rectangular and italics represent the metabolites and enzymes measured in current study, which are involved in the glycolysis, Krebs' cycle, and AMP-activated protein kinase (AMPK) (dashed rectangular) pathways identified in jejunal mucosa. 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 3 treatments (Control, T1, T2) when there is no significant interaction between treatment and days. Control = basal diets containing 1.59% L-Ala (iso-nitrogen); T1 = basal diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp; T2 = energy deficiency diet containing 1% L-Gln, 0.5% L-Glu, and 0.1% L-Asp. 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.

pathway levels on d 21-post weaning. Previous studies have demonstrated that Gln could regulate enterocyte growth under normal energy conditions through activating the mTOR pathway but inhibiting the AMPK pathway (Yi et al., 2015; Zhai et al., 2015). Days 2 to 5 after weaning is the most severe stage during the weaning-period (Wang et al., 2015b). Piglets on d 2 to 5 post-weaning require more energy to rebuild the impaired intestinal mucosal barrier (Marion et al., 2002). Low energy may compromise intestinal mucosal structure, decrease the digestion and absorption function, and increase the susceptibility of weaning piglets to stress (Chen et al., 2017b). It is possible that Gln, Glu, and Asp could restore the small intestinal energy homeostasis via replenishing the 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 (Fig. 4). 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 (Zabielski et al., 2008; Wang et al., 2016b). Asp and AKG have been proven to improve energy status through modulating the AMPK pathway in piglets under stress (Hou et al., 2011; Pi et al., 2014). However, the major limitation of this study is the absence of a low energy control. The data of the current study cannot suggest that Gln, Glu, and Asp could restore the energy homeostasis in piglets under low energy feed. Further study should consider setting up a low energy control and higher amino acids dosages. Whether increasing the amino acids dosage based on this study could enhance their efficacy on the energy regulation in piglets on d-5 post-weaning remains to be verified. Higher dosages of amino acids might improve the energy imbalance induced by low energy intake via replenishing TCA instead of activating the AMPK pathway. However, the nitrogen balance and ammonia toxicity should be taken into account when increasing the levels of amino acids. Access to amino acids may greatly affect food intake, impair systemic NO synthesis or increase the concentration of plasma ammonia, which is associated with serious tissue injuries (Wu, 2017). Ammonia could interfere with the metabolism and integrity of intestinal mucosa cells through increasing nucleic synthesis (Chen et al., 2017a). Additionally, because the single Glu, Gln or Asp supplementation was not set up in the current study, the synergistic effect of the 3 amino acids were not fully discussed, which needs to be improved on in the following-up studies.

5. Conclusion

The present result shows that supplementation of Gln, Glu, and Asp in a normal energy diet can promote the growth performance of weaning piglets 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. At normal energy supplementation, 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 were raised 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 insufficient energy intake in weaning piglets.

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.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Appendix

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2021.07.009.

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