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Original Research Article

Interaction between energy level and starch:fat ratio on intestinal energy metabolism of layer pullets

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ABSTRACT

During the growing period, the gastrointestinal tract of layer pullets is not yet well developed and may be susceptible to dietary energy level. The energy level and composition might impact the intestinal energy metabolism of layer pullets. To test this hypothesis, a total of 480 "Jing Tint 6" layer pullets were used in an 8-week study and allocated to 4 groups, each consisting of 8 replicates, with 15 birds per replicate. Pullets were treated with low or high starch:fat ratios (LS, 10:1; HS, 20:1) in a 2 × 2 factorial arrangement with regular energy (RE, 11.85 and 11.68 MJ/kg for birds from 6 to 10 weeks of age and 11-14 weeks of age, respectively) or low energy (LE, 0.55 MJ/kg lower than RE) levels. A significant interaction ($P < 0.05$) showed that HS increased glandular stomach weight and the jejunal villus length to crypt depth ratio (VCR) in LE diets, but decreased these parameters in RE diets. Dietary energy reduction impaired energy metabolism in the ileum ($P < 0.05$) mainly via decreasing the gene expression of enzymes involved in the tricarboxylic acid (TCA) cycle (α-ketoglutarate dehydrogenase complex [*α-KGDH*]; isocitrate dehydrogenase (NAD (+) [*IDH*] catalytic; citrate synthase [*CS*]) and adenosine triphosphate (ATP) synthesis, reducing contents of phosphoenolpyruvate (PEP) and adenylate energy charges (AEC) and down-regulating the adenosine monophosphate-activated protein kinase (AMPK) pathway. HS stimulated AMPKα phosphorylation, increased protein abundance of peroxisome proliferator activated-receptor gamma coactivator 1α (PGC1α) and improved contents of amino acids (aspartate, glutamate, glutamine, alanine and threonine) and malate in the ileum regardless of energy levels ($P < 0.05$). By an interaction ($P < 0.05$), the transition from LS to HS diets up-regulated ileal gene expression of *AMPKα1* and decreased content of adenosine monophosphate (AMP), accompanied by higher AEC but only in birds fed with LE diets. Collectively, these results suggest that low energy feeding may not be enough for maintaining intestinal energy homeostasis in layer pullets and emphasizes the importance of a relatively high starch:fat ratio in restoring energy metabolism in the ileum.

1. Introduction

Dietary energy is the main factor affecting body weight and flock uniformity of layer pullets. The feed costs of layer pullets, especially the energy ingredients account for a large economic expense (Hassan et al., 2020). Reducing energy level could be an effective approach to lowering feed costs and ensuring optimal body weight of layer pullets. An appropriate low energy diet could improve flock uniformity and laying performance of laying hens from 6 to 72 weeks of age (Lu et al., 2023). However, other studies have demonstrated that young pullets fed energy-reduced diets may not be able to satisfy the energy requirements for organ development, especially intestinal growth (Scappaticcio et al., 2022; Perez-Bonilla et al., 2012). Also, energy reduction exerted negative effects on nutrient digestibility (Fang et al., 2019) and energy metabolism (Liu et al., 2024), compromising the integrity of intestinal mucosa (Wang et al., 2022). It is reasonable to speculate

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that nutritional intervention targeting energy metabolism could regulate the intestinal health of layer pullets, thus ensuring optimal body weight and maximum laying performance during later phases.

Energy-rich feeds of birds are typically high in starch and fat. Dietary energy density, especially starch and fat composition, could make a difference in nutrient and energy utilization of poultry (Barekatain et al., 2021; Wu et al., 2019). Dietary starch and fat differ in intestinal digestion and absorption due to their chemical characteristics (Moss et al., 2018; Palomar et al., 2023). Dietary corn starch is digested more readily and rapidly than corn grain, enhancing nutrient utilization (Moss et al., 2018). Undigested starch could provide energy substrates such as short chain fatty acids via microbial fermentation in the hindgut (Gao et al., 2022). However, the fermented products have a lower energy efficiency compared with digested ones, resulting in a contradictory effect (Tan et al., 2021). In general, metabolizable energy (ME) comprises energy allocated for growth and production, as well as energy lost as heat. Starch has higher heat energy loss during digestion and absorption compared to fat (Sharma et al., 2021), resulting in a relatively lower net energy value in laying hens (Barzegar et al., 2019) and broilers (Carré et al., 2014). This indicates that the dietary composition of starch and fat is crucial for energy partitioning between adenosine triphosphate (ATP) production and intestinal heat energy loss (Smith et al., 1978). In the literature it has been reported that dietary starch:fat ratios have varying influences on whole-body energy metabolism (Gao et al., 2023), intestinal morphology (Adebowale et al., 2019), concentrations of amino acids in portal circulation (Yin et al., 2019), short-chain fatty acids (Granstad et al., 2021) and colonic health (Nielsen et al., 2015) in monogastric animals. Nevertheless, the overall regulation of intestinal energy metabolism in response to different starch:fat ratios is still not clear.

The intestinal tract is a highly dynamic organ that requires an enormous amount of ATP for digestion, absorption, and rapid renewal of epithelium (Hunter and Mitchell, 2002; Kelly and McBride, 1990). The intestine can sense and regulate glucose, amino acids and fatty acids, whose cell-specific oxidation produces biological energy (Dyer et al., 2007). The tricarboxylic acid (TCA) cycle is a central hub for energy generation, linking the metabolic pathways of these nutrients (Arnold et al., 2022). The regulation of key enzymes and metabolites involved in the TCA cycle has been extensively studied to facilitate energy production in poultry (Mon et al., 2020; Zhou et al., 2023). Adenosine monophosphate-activated protein kinase (AMPK), as an energy sensor, is responsible for regulating energy balance and nutrient intake in the intestine. Activation of AMPKα results in an increase in peroxisome proliferator activated-receptor gamma coactivator 1α (PGC1α), which could further stimulate mitochondrial biogenesis and promote the differentiation of intestinal epithelium cells (Sun et al., 2022). Also, the AMPK pathway has been supposed to regulate nutrient absorption (Dengler et al., 2017). Nutritional strategies have been well documented to enhance energy metabolism via regulating the AMPK-PGC1α pathway (Elamin et al., 2013; Wang et al., 2022; Zhang et al., 2021). It is worth noting that dietary corn starch supplementation has been found to promote the AMPK pathway in response to insufficient energy supply in broilers (Liu et al., 2020).

The hypothesis of this research is that the change in dietary energy level could regulate the intestinal energy metabolism of layer pullets, and this effect could be modified by different starch:fat ratios. Hence, in the present study, the interactive effects of energy levels and starch:fat ratios on gastrointestinal mass, intestinal morphology, the AMPK pathway, energy metabolites and enzymes involved in the TCA cycle in the small intestine were determined from layer pullets at 14 weeks of age.

2. Materials and methods

2.1. Animal ethics statement

All animal care and use procedures were evaluated and approved by the Animal Care and Experiment Committee of China Agricultural University (AW51304202-1-2).

2.2. Diets

A 2 × 2 factorial design resulted in 4 dietary treatments with either low or regular energy levels (LE, RE) and low or high starch:fat ratios (LS, HS). Diets were formulated by using typical ingredients including corn, soybean meal, soybean oil and wheat bran. The energy levels of regular energy diets are 11.85 and 11.68 MJ/kg (as-fed basis) for growing (6-10 weeks of age) and developing (11-14 weeks of age) stages of layer pullets, recommended by commercial feeding management of "Jing Tint 6" layer pullets. Energy levels of LE diets were 0.55 MJ/kg lower than the recommended energy levels, and wheat bran was used to dilute energy levels. Common corn starch was purchased from Qinhuangdao Lihua Starch Co., Ltd. (Qinhuangdao, China) and its nutrients complied with food requirements (National recommended standard of China, GB/T 8885−2017). Soybean oil was obtained from Techlex Co. Ltd. (Zhuozhou, China). Experiment diets were formulated to contain either 40% or 45% starch by supplementation of corn starch. The 40% starch diets contained 4% fat and the 45% starch diets contained 2.1% fat, with iso-protein levels across all diets (17.0% and 15.5%, as-fed basis for broilers at 6-10 weeks of age and 11-14 weeks of age, respectively). The formulations generated different starch:fat ratios at 10:1 and 20:1 for LS and HS, respectively. Coccidiostats, antibiotics or growth promoters were not contained in the experimental diets. The composition and nutrient specifications of the experimental diets are presented in Table 1. The nutrient specifications were analyzed using AOAC official methods (2016) to determine the concentrations of crude protein (method 954.01), crude fat (method 920.39) and calcium (method 927.02) on a dry matter basis. In detail, the content of nitrogen was determined by using the Kjeldahl procedure (KT 200,101 Kjeltec distillation unit, Hilloeroed, Denmark) and protein was then calculated as N × 6.25. Quantification of crude fat was conducted using a Soxhlet extraction apparatus (Wiggens, Germany) using petroleum ether. Moreover, calcium content was measured by dry ashing. The starch content was determined by using a commercial kit (A148-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, the starch was decomposed into glucose by acid hydrolysis and then glucose was quantified using colorimetric method.

2.3. Animal management and growth performance

A total of 480 "Jing Tint 6" at 6 weeks of age were weighted and randomly assigned into 4 groups with 8 repeats each. Birds were placed in hen cages (40 cm × 37 cm × 34 cm), with 3 pullets per cage and 5 cages per repeat. Birds were vaccinated against main diseases such as Newcastle disease, infectious bronchitis and Marek's disease, following standard commercial practices in Beijing. During the experiment, diets were offered in the form of mash twice daily, at 09:00 and 15:00. A 10L:14D lighting program with approximately 5 to 10 lx was used, and the temperature of the shed was maintained at 18 to 24 °C throughout the whole trial.

Feed intake was determined at 10 weeks of age and 14 weeks of age, and the average daily feed intake (ADFI) was calculated. The feed conversion ratio (FCR) was calculated by dividing feed intake by body weight gain. The body weight (BW) of each bird was

Table 1
Composition and nutrient levels of experiment diets.[1]

Item	6–10 weeks of age				11–14 weeks of age			
	LELS	LEHS	RELS	REHS	LELS	LEHS	RELS	REHS
Ingredients (as-fed basis,%)								
Corn	58.00	42.95	63.36	52.64	58.43	44.49	64.54	54.90
Soybean meal	23.31	27.52	24.66	28.04	18.43	22.55	19.76	22.99
Corn starch		16.22		13.01		15.52		11.71
Soybean oil	0.82		1.09		0.90		0.90	
Wheat bran	14.07	9.60	7.10	2.59	18.07	13.37	10.65	6.31
Dicalcium phosphate	1.66	1.62	1.66	1.64	1.80	1.80	1.80	1.85
Choline chloride (50%)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Limestone	1.21	1.20	1.22	1.20	1.40	1.37	1.40	1.34
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Mineral and vitamin premix[2]	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
DL-Methionine	0.14	0.15	0.14	0.14	0.15	0.16	0.15	0.16
L-Lysine hydrochloric acid	0.05		0.03		0.08		0.06	
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Calculated nutrient content (as-fed basis, %)[3]								
Metabolizable energy, MJ/kg	11.30	11.30	11.85	11.85	11.13	11.13	11.68	11.68
Crude protein	17.00	17.01	17.00	17.00	15.50	15.50	15.50	15.50
Starch	40.00	45.03	41.05	45.45	39.98	45.86	40.29	45.13
Crude fat	4.05	2.04	3.89	2.24	3.99	2.08	3.97	2.24
Calcium	0.90	0.90	0.90	0.90	0.98	0.98	0.98	0.98
Crude fiber	3.79	3.29	3.36	2.91	3.89	3.39	3.43	3.00
Neutral detergent fiber	13.77	11.29	11.87	9.67	14.63	12.15	12.63	10.56
Acid detergent fiber	5.49	4.95	4.93	4.44	5.52	4.97	4.92	4.44
Lignin	0.87	0.88	0.86	0.88	0.80	0.78	0.78	0.81
Lysine	0.96	0.96	0.96	0.96	0.85	0.85	0.85	0.85
Digestible lysine	0.85	0.85	0.85	0.85	0.75	0.75	0.76	0.75
Methionine	0.42	0.42	0.42	0.42	0.41	0.41	0.41	0.41
Digestible methionine	0.40	0.40	0.40	0.40	0.39	0.39	0.39	0.39
Methionine + cysteine	0.73	0.71	0.72	0.71	0.70	0.69	0.70	0.69
Tryptophan	0.19	0.19	0.19	0.19	0.17	0.18	0.17	0.17
Threonine	0.66	0.66	0.66	0.66	0.58	0.60	0.59	0.60
Digestible threonine	0.55	0.55	0.55	0.55	0.48	0.49	0.49	0.50
Available phosphorus	0.39	0.37	0.37	0.37	0.41	0.41	0.40	0.40
Starch:fat ratio	9.88	22.07	10.55	20.29	10.02	22.05	10.15	20.15
Analyzed nutrient content (DM basis, %)								
Crude protein	19.22	19.24	19.34	19.19	17.19	17.33	17.20	17.38
Calcium	1.01	1.06	1.03	0.99	1.10	1.11	1.06	1.10
Starch	43.26	50.25	46.43	53.67	44.97	51.70	47.42	52.69
Crude fat	3.99	2.40	4.19	2.46	4.22	2.57	4.13	2.65
Starch:fat ratio	12.19	23.48	12.45	24.51	11.96	22.57	12.89	22.33

[1] LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6-10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch:fat ratio diet (20:1).
[2] Mineral premix provided the following per kg of diets: Cu 8 mg, Zn 75 mg, Fe 80 mg, Mn 100 mg, Se 0.15 mg, I 0.35 mg. Vitamin premix provided the following per kg of diets: VA 9500 IU, VD 362.5 μg, VE 30 IU, VK_3 2.65 mg, VB_1 2 mg, VB_2 6 mg, VB_6 6 mg, VB_{12} 0.025 mg, biotin 0.0325 mg, folic acid 1.25 mg, pantothenic acid 12 mg, nicotinic acid 50 mg.
[3] Nutrient content was calculated using software VF123 for 2022 (Jinmu Times Technology Co., Ltd., Beijing, China), based on the 31st version of Tables of Feed Composition and Nutritive Values in China (China feed database, 2020).

measured to calculate the flock uniformity, expressing as the percentage of birds within 10% of average body weight relative to the total number of birds.

2.4. Gut sampling and intestinal morphology

One bird per replicate was selected and slaughtered after electrical stunning at 14 weeks of age. The entire intestine was collected and carefully straightened, and the length of each section was measured. The contents of the glandular stomach, gizzard, jejunum and ileum were removed while the glandular stomach and gizzard were weighed. The ileal mucosa was collected in 2 mL centrifuge tubes for metabolomic profile analysis. One piece of 1 cm length of jejunal and ileal tissues from the midpoint were fixed in 4% formaldehyde for morphological analysis. Other pieces of jejunum and ileum from the midpoint were immediately immersed in liquid nitrogen and then stored at −80 °C for further analysis. The fixed jejunal and ileal segments were embedded in paraffin before staining with hematoxylin-eosin to visualize intestinal morphology. The villus height and crypt depth were determined by Image-Pro Plus software (Version 6.0, Media Cybernetics, USA), and the villus height to crypt depth (VCR) was calculated.

2.5. RNA isolation and quantitative real time-PCR (qRT-PCR) analysis

Approximately 1 g tissue was cut from the frozen sample and homogenized in 1 mL Trizol reagent (Genstar, Beijing, China) to extract total RNA. The concentration and purity of extracted RNA were verified at 260/280/230 nm by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Then cDNA was synthesized from 1000 ng total RNA following the manufacturer's protocol with a High Capacity cDNA Reverse Transcription Kit (Genstar, Beijing, China). Quantitative real time-PCR was run on a Step Two Plus System, using a qRT-PCR kit (Genstar, Beijing, China) according to the manufacturer's protocol. The mRNA expression in the small intestine was evaluated using the following conditions: 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s

and 60 °C for 30 s. Table 2 displays the sequence of primers that was used to amplify target genes. The target gene expression normalized by housekeeping gene of β-actin was calculated and analyzed by the $2^{-\triangle\triangle Ct}$ method.

2.6. Western blotting

About 30 mg ileal tissue was homogenized in RIPA (Catalogue #P0013B, Beyotime Biotechnology, Shanghai, China), and then the mixture was centrifuged at 12,000 × *g* for 10 min to obtain the supernatant. The supernatant fluid was adjusted to an equal concentration (2.5 mg/mL) by using bovine serum albumin (BSA) as standard (Catalogue #P0010, Beyotime Biotechnology, Shanghai, China). The samples were diluted with 6 × sample loading buffer and were heated at 99 °C for 10 min in a metal bath, and then were cooled down for Western blot analysis. In brief, the protein was isolated from 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then was transferred to the PVDF membrane (Catalogue #IPVH00010, Merck KGaA, Darmstadt, Germany) for immunoblotting. The primary antibodies adopted in this study were AMPKα (1:500; Catalogue #AF6195, Beyotime Biotechnology), phosphorylated AMPKα (1:500; Catalogue #AF5908, Beyotime Biotechnology), PGC1α (1:500; Catalogue #AF7736, Beyotime Biotechnology), ATP synthase α subunit (ATP5A, 1:500; Catalogue #K108946P, Solarbio Science & Technology Co., Ltd. Beijing, China) and β-actin (1:2,000; Catalogue #P30002S, Abmart, Shanghai, China). The secondary antibody used in this study was goat anti-rabbit immunoglobulin G horseradish peroxidase (IgG HRP, 1:5000; Catalogue #C31460100, Thermo Fisher Scientific, Waltham, USA). The images were detected by ECL western blotting substrate (Catalogue #PE0010, Solarbio Science & Technology Co., Ltd. Beijing, China) and visualized using the Chemibioimaging System. The gray values of protein bands were quantified by ImageJ software. AMPKα, PGC1α and ATP5A were standardized using the protein abundance of β-actin, and phosphorylated AMPKα (p-AMPKα) was standardized using the protein abundance of total AMPKα.

Table 2
Primer sequences used in the quantitative real time-PCR.[1]

Item	Primer sequence (5′ to 3′)	GenBank accession No.
SDHA	F: ATTCCCGTTTTGCCTACGGT	NM_001277398.1
	R: GGGAGTTTGCTCCAAGACGA	
α-KGDH	F: TTCAAGCACAGCCCAACGTA	NM_001031382.2
	R: GCCCGTAAAATCCGACGTTT	
IDH3A	F: GACCAGAAGTTTTAGTAGTGCTGT	NM_001005808.3
	R: AGGTTTTACGCAGCAGCAGA	
IDH3B	F: GTCCTTACGAGTGGCTGGTT	XM_046940299.1
	R: CTGGGCAAGAACAGGAGGAG	
CS	F: GGAACGGGCGTTGTTTCGG	XM_046905018.1
	R: TGCGGGCGCTGATTCATTA	
ATP5A	F: GGCAATGAAACAGGTGGCAG	XM_429118.5
	R: GGGCTCCAGCTTGTCTAAGTGA	
AMPKα1	F: CACAGCAGTGCCAAGAGAGA	NM_001039603.2
	R: GCTGTGCTGCTACTACGCTA	
AMPKα2	F: CACCTTCGGCAAAGTCAAGG	NM_001039603.2
	R: GAAGAAGTCTGTTGGCGTGC	
PGC1α	F: AGAAAGGGTCTCGTTGCTGC	NM_001006457.2
	R: AGCACACTCGATGTCACTCC	
ACC	F: CACCTCCCACCCAAACAGAA	NM_205505.2
	R: AACGTGCGGAAAGAGACCAT	
ESRRα	F: ATGATCGGCTTAGTCTGGCG	NM_205183.2
	R: GCAGCAGTAGCCAGTAGCAT	
NRF1	F: GTGTCCCTCATCCAGGTTGG	NM_001030646.2
	R: TGCGACGGTAACTGTGGTAG	
NRF2	F: GCGCAGTAAAGAAGGAAGAGC	NM_001396902.1
	R: TTGAAACAGGCACTGCAGGA	
TFAM	F: AACCTGAGTTATGCTGCTGT	NM_204100.2
	R: GTCACATTTCCTGCGCCTTC	
β-Actin	F: CAACACAGTGCTGTCTGGTGGTAC	XM_027015741.1
	R: CTCCTGCTTGCTGATCCACATCTG	

SDHA = succinate dehydrogenase A; *α-KGDH* = α-ketoglutarate dehydrogenase complex; *IDH3A* = isocitrate dehydrogenase (NAD (+)) catalytic subunit α; *IDH3B* = isocitrate dehydrogenase (NAD (+)) non-catalytic subunit β; *CS* = citrate synthase; *ATP5A* = ATP, synthase α subunit; *AMP*K = adenosine monophosphate-activated protein kinase; *PGC1α* = peroxisome proliferator activated-receptor gamma coactivator 1α; *ACC* = acetyl CoA carboxylase; *ESRRα* = estrogen-related receptor α; *NRF* = nuclear respiratory factor; *TFAM* = transcription factor A, mitochondrial.

[1] These primers have been specifically designed for this experiment at the National center for Biotechnology Information (NCBI, Bethesda, Maryland, USA).

2.7. Metabolomic profiling

The metabolomic profile related to energy metabolism was assessed by using ultra performance liquid chromatography (UPLC)-mass spectrometry (MS)/MS according to a previous study (Wang et al., 2022). Briefly, 50 mg ileal mucosa was homogenized in 100 μL double-distilled H_2O (ddH_2O) and mixed with 500 μL pre-cooled methanol/ddH_2O (7:3, vol:vol), then vortexed for 3 min. Next, the mixture was centrifuged at 14,000 × *g* for 10 min at 4 °C. About 300 μL supernatant was drawn and incubated at −20 °C for 30 min. After centrifuging at 14,000 × *g* for 10 min again, the supernatant was suspended for UPLC-MS/MS analysis. The UPLC (Waters, Milford, USA) was equipped with a 2.1 mm × 100 mm ACQUITY UPLC BEH Amide column (internal diameter 1.7 μm; Waters, Milford, USA), and the column was heated to 45 °C before analysis. The mobile phase for UPLC analysis included two solutions: (A) H_2O (10 mmol/L ammonium acetate and 0.3% ammonia) and (B) acetonitrile/H_2O (9:1, vol:vol). The spectra of MS/MS (QTRAP 6500+, SCIEX, Framingham, USA) was set as follows: temperature of electrospray ionization, 450 °C; curtain gas, 35 psi; positive ion voltage floating, 5500 V; negative ion voltage floating, −4500 V. The concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) obtained by these procedures were used to calculate total adenine nucleotide (TAN) and adenylate energy charges (AEC) as follows (David et al., 1978):

$$TAN = ATP + ADP + AMP;$$

$$AEC = (ATP + 0.5\ ADP)/(ATP + ADP + AMP).$$

2.8. Statistical analysis

Each replicate or individual bird was considered as an experiment unit. All data of the experiment were performed by two-way analysis of variance using SPSS 24.0 (SPSS Inc., Chicago, USA), with "energy level" and "starch:fat ratio" as main effects. The statistical model used was as follows:

$$Y_{ij} = \mu + E_i + S_j + R_{ij} + \varepsilon_{ij},$$

where Y_{ij} is the observation of dependent variables; μ is the overall mean; E_i is the "energy level" effect; S_j is the "starch:fat ratio" effect, R_{ij} represents the interactive effect between the two factors, and ε_{ij} is the residual error for the observation. Meanwhile, differences among the 4 treatments were evaluated using one-way ANOVA and Duncan's multiple comparisons when a significant interaction was observed. The variation of differential metabolites in metabolomic profiles was analyzed by fold change (FC, FC ≥ 2 or FC ≤ 0.5) and variable importance in projection (VIP, VIP >1). Results are

Table 3
Daily feed intake, body weight, feed conversion ratio and flock uniformity of layer pullets at 6 to 14 weeks of age.[1]

Item	Average daily feed intake, g/d			Body weight, kg		Feed conversion rate, g/g			Flock uniformity,[2] %	
	6–10 weeks of age	11–14 weeks of age	6–14 weeks of age	10 weeks of age	14 weeks of age	6–10 weeks of age	11–14 weeks of age	6–14 weeks of age	10 weeks of age	14 weeks of age
Treatment										
LELS	49.10	55.37^{a}	52.23^{a}	0.811^{a}	1.076^{a}	3.20	5.87	4.20	87.96	95.47
LEHS	49.17	53.31^{b}	51.24^{ab}	0.787^{b}	1.049^{b}	3.29	5.71	4.20	89.33	91.83
RELS	46.81	52.48^{b}	49.65^{c}	0.784^{b}	1.066^{ab}	3.10	5.24	3.95	82.14	94.64
REHS	47.80	53.29^{b}	50.54^{bc}	0.789^{b}	1.071^{a}	3.18	5.32	4.05	86.54	91.96
Pooled SEM	0.302	0.293	0.238	0.0034	0.0037	0.019	0.059	0.026	1.559	1.174
Energy level effect										
LE	49.13^{a}	54.34^{a}	51.74^{a}	0.799^{a}	1.062	3.25^{a}	5.79^{a}	4.20^{a}	88.64	93.65
RE	47.31^{b}	52.88^{b}	50.10^{b}	0.787^{b}	1.068	3.14^{b}	5.28^{b}	4.00^{b}	84.34	93.30
Starch:fat ratio effect										
LS	47.96	53.92	50.94	0.798	1.071	3.15^{b}	5.55	4.07	85.05	95.06
HS	48.48	53.30	50.89	0.788	1.060	3.23^{a}	5.51	4.13	87.93	91.90
***P*-value**										
Energy level	0.001	0.004	<0.001	0.047	0.402	0.001	<0.001	<0.001	0.179	0.887
Starch to fat ratio	0.320	0.190	0.114	0.126	0.889	0.010	0.628	0.171	0.363	0.198
E × S[3]	0.381	0.005	0.025	0.016	0.011	0.936	0.123	0.184	0.631	0.842

[a-c]Mean values within a column with different superscript letters are significantly different ($P < 0.05$), $n = 8$.
[1] LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6-10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch: fat ratio diet (20:1).
[2] Flock uniformity is calculated as the percentage of birds within 10% of average body weight to total numbers of birds.
[3] E × S = the interaction between energy level and starch:fat ratio.

expressed as mean and pooled standard error of the mean (SEM) and considered as significant at $P < 0.05$.

3. Results

3.1. Growth performance

As shown in Table 3, significant interactions were observed ($P < 0.05$) between energy level and starch:fat ratio for the ADFI (from 11–14 weeks of age and 6-14 weeks of age) and BW (at 10 and 14 weeks of age), and the highest values were found in the LELS diet. Birds fed low energy diets tended to increase their feed intake to meet energy requirements. By an interaction, birds fed the LELS diet showed compensatory BW ($P = 0.016$) at 10 weeks of age, while those fed the LEHS diet exhibited a reduction in BW ($P = 0.011$) at 14 weeks of age. In addition, the FCR (from 6–10 weeks of age, 11-14 weeks of age and total period) were increased in birds fed with LE diets ($P < 0.05$) without interactions.

3.2. Gastrointestinal mass and intestinal morphology

The responses of small intestinal length, stomach weight, jejunal and ileal morphology are shown in Table 4. Compared with a low starch:fat ratio, the high starch:fat ratio decreased jejunal length ($P = 0.071$) regardless of energy levels. Compared with regular energy, energy reduction tended to reduce duodenum length ($P = 0.099$) of birds. A treatment interaction was observed for glandular stomach weight ($P = 0.008$) as the high starch:fat

Table 4
Intestinal length, stomach weight and intestinal morphology of layer pullets at 14 weeks of age.[1]

Item	Intestinal length, cm			Stomach weight,[2] %		Jejunum[3]			Ileum[3]		
	Duodenum	Jejunum	Ileum	Glandular stomach	Gizzard	Villus length, μm	Crypt depth, μm	VCR	Villus length, μm	Crypt depth, μm	VCR
Treatment											
LELS	15.48	54.16	45.69	0.46^{ab}	2.33	754.53	115.43	6.58^{b}	640.17	92.83	6.89
LEHS	16.38	48.94	45.81	0.50^{a}	2.24	912.25	109.86	8.30^{a}	507.30	89.56	5.70
RELS	16.50	52.90	48.29	0.47^{a}	2.14	855.90	97.70	8.79^{a}	554.89	83.43	6.79
REHS	17.63	51.00	47.00	0.42^{b}	2.09	778.59	105.90	7.36^{ab}	554.65	90.46	6.23
Pooled SEM	0.345	0.969	1.206	0.010	0.039	30.684	2.430	0.285	22.573	2.245	0.248
Energy level effect											
LE	15.93	51.55	45.75	0.48	2.28^{a}	833.39	112.64^{a}	7.44	573.74	91.30	6.34
RE	17.06	51.95	47.64	0.45	2.11^{b}	817.25	101.80^{b}	8.07	554.77	86.94	6.51
Starch:fat ratio effect											
LS	15.99	53.53	46.99	0.47	2.23	805.22	106.57	7.69	597.53	88.13	6.84
HS	17.00	49.97	46.41	0.46	2.16	845.42	107.88	7.83	530.98	90.04	5.98
***P*-value**											
Energy level	0.099	0.835	0.456	0.053	0.042	0.790	0.023	0.224	0.670	0.358	0.668
Starch:fat ratio	0.140	0.071	0.818	0.666	0.366	0.509	0.772	0.781	0.142	0.681	0.086
E × S[4]	0.867	0.388	0.780	0.008	0.825	0.061	0.136	0.004	0.143	0.267	0.526

VCR = villus length to crypt depth ratio.
[a,b]Mean values within a column with different superscript letters were significantly different ($P < 0.05$), $n = 8$.
[1] LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6-10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch:fat ratio diet (20:1).
[2] The percentage of the glandular stomach and gizzard weight to the live body weight of birds at 14 weeks of age.
[3] The villus length and crypt depth were measured 8 replicates per tissue.
[4] E × S = the interaction between energy level and starch:fat ratio.

Table 5
Treatment effects on relative gene expression of enzymes related to the TCA cycle in the jejunum and ileum of layer pullets at 14 weeks of age.[1]

Item	Jejunum					Ileum				
	SDHA	α-KGDH	IDH3A	IDH3B	CS	SDHA	α-KGDH	IDH3A	IDH3B	CS
Treatment										
LELS	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
LEHS	1.06	0.77	0.84	1.01	1.01	1.00	0.78	0.82	0.74	0.48
RELS	1.26	0.75	1.15	1.15	1.07	1.08	1.48	1.10	2.32	2.02
REHS	1.15	0.53	0.92	1.09	0.95	1.09	1.71	1.02	1.96	2.01
Pooled SEM	0.075	0.111	0.082	0.403	0.088	0.033	0.109	0.170	1.336	0.942
Energy level effect										
LE	1.03	0.87	0.92	1.00	1.01	1.00	0.89[b]	0.91[b]	0.87[b]	0.74[b]
RE	1.20	0.64	1.03	1.12	1.01	1.08	1.60[a]	1.06[a]	2.14[a]	2.02[a]
Starch:fat ratio effect										
LS	1.13	0.85	1.07	1.07	1.03	1.04	1.24	1.05[a]	1.66	1.51
HS	1.11	0.65	0.88	1.04	0.98	1.04	1.25	0.92[b]	1.35	1.25
***P*-value**										
Energy level	0.277	0.288	0.514	0.473	1.000	0.245	0.001	0.005	0.006	<0.001
Starch:fat ratio	0.881	0.328	0.263	0.871	0.780	0.949	0.975	0.012	0.482	0.290
E × S[2]	0.577	0.977	0.842	0.822	0.724	0.964	0.226	0.314	0.917	0.313

SDHA = succinate dehydrogenase A; *α-KGDH* = α-ketoglutarate dehydrogenase complex; *IDH3A* = isocitrate dehydrogenase (NAD (+)) catalytic subunit α; *IDH3B* = isocitrate dehydrogenase (NAD (+)) non-catalytic subunit β; *CS* = citrate synthase.
[a,b]Mean values within a column with different superscript letters are significantly different ($P < 0.05$), $n = 8$.
[1] LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6-10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch:fat ratio diet (20:1).
[2] E × S = the interaction between energy level and starch:fat ratio.

ratio significantly decreased glandular stomach weight in birds offered regular energy diets but had no influence in birds fed low energy diets. Heavier glandular stomach ($P = 0.053$) and gizzard ($P = 0.042$) were observed in birds fed low energy diets compared with those fed regular energy diets irrespective of starch:fat ratios. Meanwhile, a significant interaction was observed for jejunal VCR ($P = 0.004$) as the high starch:fat ratio significantly increased VCR in low energy diets, but numerically suppressed it in regular energy diets. In addition, dietary high starch:fat ratio numerically depressed VCR in the ileum irrespective of energy levels ($P = 0.086$).

3.3. Enzymes involved in TCA cycle

Effects of dietary treatments on jejunal and ileal gene expression of enzymes involved in the TCA cycle are presented in Table 5. Treatment interactions were not observed for gene expression of TCA cycle enzymes either in the jejunum or ileum ($P > 0.05$). As a main effect, energy reduction depressed relative gene expression of α-ketoglutarate dehydrogenase complex (*α-KGDH*), isocitrate dehydrogenase (NAD (+)) catalytic subunit α (*IDH3A*), non-catalytic subunit β (*IDH3B*) and citrate synthase (*CS*) in the ileal tissue ($P < 0.05$). In addition, regardless of energy levels, a high starch:fat ratio decreased ileal gene expression of *IDH3A* ($P = 0.012$).

3.4. Metabolites of glycolysis and TCA cycle

As shown in Table 6, significant interactions were observed ($P < 0.05$) between energy levels and starch:fat ratios for fructose-6-phosphate (F-6-P) and glucose-6-phosphate (G-6-P) in ileal mucosa. By interaction, low starch:fat ratio increased F-6-P ($P = 0.011$) and G-6-P ($P = 0.013$) in low energy diets but had no

Table 6
Treatment effects on metabolites of glycolysis and TCA cycle in the ileal mucosa of layer pullets at 14 weeks of age.[1]

Item	Glycolysis metabolites						TCA cycle metabolites			
	PEP, mg/g	DHAP, μg/g	F-6-P, μg/g	G-6-P, μg/g	F-1,6-P, μg/g	α-KG, μg/g	Malate, μg/g	Oxaloacetate, μg/g	Isocitrate, ng/g	Pyruvate, μg/g
Treatment										
LELS	40.87	3.79	23.50[a]	89.63[a]	7.12	1.09	20.19	0.39	27.95	1.61
LEHS	151.17	2.62	7.15[b]	39.59[b]	6.03	1.11	21.39	0.36	23.35	1.63
RELS	215.36	2.60	6.32[b]	38.09[b]	2.51	1.03	17.80	0.33	31.88	1.65
REHS	235.67	2.61	8.18[b]	55.49[ab]	4.93	1.08	27.71	0.36	35.77	2.81
Pooled SEM	32.081	0.251	2.109	7.205	0.832	0.031	1.348	0.015	2.142	0.249
Energy level effect										
LE	96.02[b]	3.21	15.33[a]	64.61	6.68	1.10	20.79	0.38	25.39	1.62
RE	225.52[a]	2.61	7.25[b]	46.79	3.72	1.06	22.76	0.34	34.04	2.23
Starch:fat ratio effect										
LS	128.12	3.20	14.91[a]	63.86	4.81	1.06	18.99[b]	0.36	29.91	1.63
HS	193.42	2.62	7.67[b]	47.54	5.37	1.09	24.55[a]	0.36	29.56	2.22
***P*-value**										
Energy level	0.043	0.230	0.021	0.165	0.095	0.492	0.418	0.250	0.059	0.224
Starch:fat ratio	0.288	0.244	0.037	0.202	0.686	0.583	0.030	0.981	0.930	0.239
E × S[2]	0.461	0.239	0.011	0.013	0.293	0.802	0.083	0.258	0.304	0.256

TCA = cycle = tricarboxylic acid cycle; PEP = phosphoenolpyruvate; DHAP = dihydroxyacetone phosphate; F-6-P = fructose-6-phosphate; G-6-P = glucose-6-phosphate; F-1,6-P = fructose-1, 6-bisphosphate; α-KG = α-ketoglutarate.
[a,b]Mean values within a column with different superscript letters were significantly different ($P < 0.05$), $n = 6$.
[1] LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6-10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch:fat ratio diet (20:1).
[2] E × S = the interaction between energy level and starch:fat ratio.

Table 7
Treatment effects on concentrations of amino acids in ileal mucosa of layer pullets at 14 weeks of age (μg/g).[1]

Item	Aspartate	Asparagine	Glutamate	Glutamine	Lysine	Alanine	Threonine	Leucine	Arginine	Serine	Tyrosine
Treatment											
LELS	193.95	39.83^{ab}	482.46	26.51	11.58	42.42	65.64	61.58	25.08	107.27	89.97
LEHS	243.04	37.49^{ab}	528.70	31.42	18.60	50.18	78.16	71.23	28.09	123.86	108.39
RELS	203.35	30.88^{b}	486.29	27.72	13.84	41.32	68.28	62.86	26.98	118.94	87.71
REHS	249.70	47.15^{a}	563.55	32.81	20.40	55.21	85.09	71.38	29.38	132.59	120.48
Pooled SEM	10.355	2.213	14.023	0.936	1.769	2.081	3.572	3.149	1.095	4.749	7.441
Energy level effect											
LE	218.49	38.66	505.58	28.97	15.09	46.30	71.89	66.41	26.59	115.56	99.18
RE	226.52	39.01	524.92	30.26	17.12	48.26	76.68	67.12	28.18	125.77	104.09
Starch:fat ratio effect											
LS	198.65^{b}	35.36	484.37^{b}	27.12^{b}	12.71	41.87^{b}	66.96^{b}	62.22	26.03	113.10	88.84
HS	246.37^{a}	42.32	546.12^{a}	32.11^{a}	19.50	52.69	81.63^{a}	71.30	28.74	128.23	114.44
***P*-value**											
Energy level	0.683	0.930	0.468	0.439	0.562	0.598	0.491	0.913	0.486	0.287	0.743
Starch:fat ratio	0.023	0.095	0.028	0.006	0.063	0.008	0.044	0.174	0.241	0.121	0.099
E × S[2]	0.945	0.030	0.560	0.957	0.947	0.413	0.757	0.932	0.893	0.876	0.633

[a,b]Mean values within a column with different superscript letters are significantly different ($P < 0.05$), $n = 6$.
[1] LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6 -10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch:fat ratio diet (20:1). The concentrations of amino acids not presented in the table are below the detection levels.
[2] E × S = the interaction between energy level and starch:fat ratio.

significant influence in regular energy diets. As main effects, low energy decreased contents of phosphoenolpyruvate (PEP) ($P = 0.043$) and isocitrate ($P = 0.059$), and improved contents of F-6-P ($P = 0.021$) and F-1,6-P ($P = 0.095$), while high starch:fat ratio increased contents of F-6-P ($P = 0.037$) and malate ($P = 0.030$) in ileal mucosa.

3.5. Concentration of amino acids in ileal mucosa

Treatment effects on amino acid contents in ileal mucosa are presented in Table 7. A treatment interaction ($P = 0.030$) was observed for concentration of asparagine (Asn) in ileal mucosa as high starch:fat ratio significantly improved Asn content in regular energy diets but had no significant influence in low energy diets. As main effects, dietary high starch:fat ratio significantly increased ($P < 0.05$) contents of aspartate (Asp), glutamate (Glu), glutamine (Gln), alanine (Ala) and threonine (Thr) in the ileal mucosa with no treatment interaction.

3.6. Contents of adenine nucleotide in ileal mucosa

Treatment effects on adenine nucleotide contents in ileal mucosa are shown in Table 8. Treatment interactions were found for AMP, AMP to ATP, ADP to ATP, TAN and AEC ($P < 0.05$). Following the transition from low to high starch:fat ratio, the AMP ($P = 0.011$), AMP to ATP ($P = 0.007$), ADP to ATP ($P = 0.044$) and TAN ($P = 0.019$) significantly decreased, and the AEC ($P = 0.020$) significantly increased but only in low energy diets. Overlooking treatment interactions, dietary energy reduction increased AMP to ATP, ADP to ATP and decreased AEC as main effects ($P < 0.05$). Additionally, birds fed with low energy diets showed lower content of ATP in ileal mucosa ($P = 0.068$) compared with those fed regular energy diets.

Table 8
Treatment effects on contents of adenine nucleotide in ileal mucosa of layer pullets at 14 weeks of age.[1]

Item	AMP, μg/g	ADP, μg/g	ATP, μg/g	AMP to ATP, g/g	ADP to ATP, g/g	TAN,[2] μg/g	AEC[3], g/g
Treatment							
LELS	19.98^{a}	6.110	2.01	10.44^{a}	3.27^{a}	28.09^{a}	0.19^{b}
LEHS	6.08^{b}	4.405	3.59	2.49^{b}	1.81^{b}	14.07^{b}	0.39^{a}
RELS	5.07^{b}	5.006	5.09	1.59^{b}	1.36^{b}	15.17^{b}	0.47^{a}
REHS	12.36^{ab}	6.294	6.18	4.00^{b}	1.56^{b}	24.83^{ab}	0.42^{a}
Pooled SEM	2.151	0.4284	0.758	1.078	0.239	2.504	0.031
Energy level effect							
LE	13.03	5.257	2.80	6.47^{a}	2.54^{a}	21.08	0.29^{b}
RE	8.72	5.650	5.63	2.80^{b}	1.46^{b}	19.99	0.44^{a}
Starch:fat ratio effect							
LS	12.52	5.558	3.55	6.01	2.31	21.63	0.34
HS	9.22	5.349	4.88	3.25	1.68	19.45	0.40
***P*-value**							
Energy level	0.266	0.649	0.068	0.045	0.011	0.818	0.006
Starch:fat ratio	0.391	0.809	0.375	0.123	0.119	0.643	0.196
E × S[4]	0.011	0.094	0.871	0.007	0.044	0.019	0.020

AMP = adenosine monophosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; TAN = total adenine nucleotide; AEC = adenylate energy charges.
[a,b]Mean values within a column with different superscript letters are significantly different ($P < 0.05$), $n = 6$.
[1] LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6-10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch:fat ratio diet (20:1).
[2] TAN = AMP + ADP + ATP.
[3] AEC = (ADP × 0.5 + ATP)/(AMP + ADP + ATP).
[4] E × S = the interaction between energy level and starch:fat ratio.

3.7. Gene and protein abundance of ATP5A

As presented in Fig. 1A, no significant difference in the protein abundance of ATP5A was observed between different diets ($P = 0.344$). However, a significant interaction between energy levels and starch:fat ratios was observed for ileal gene expression of *ATP5A* ($P = 0.040$) as low starch:fat ratio significantly decreased *ATP5A* in low energy diets but had no significant influence in regular energy diets (Fig. 1B). As main effects, low energy reduced the ileal gene expression of *ATP5A* ($P = 0.009$) compared with regular energy diets.

3.8. Metabolomic profiling of ileal mucosa

The mucosal metabolomic profiles related to energy metabolism are presented in Figs. 2 and 3. The two principal components (PC) of PCA were comparable between energy levels and starch:fat ratios (Fig. 2A and B). The heatmap showed differential energy metabolites in ileal mucosa (Fig. 2A), implying that layer pullets fed with regular energy diets had greater abundances of ATP and PEP, and had lower abundances of uridine monophosphate (UMP), F-6-P and glucose-1-phosphate (G-1-P). Moreover, the volcano plot showed that there were no significant differential metabolites between low and high starch:fat ratio diets (Fig. 2B). As shown in Fig. 3A and B, the PC1 of PCA indicated that the mucosal energy metabolites between LELS and LEHS were more dispersed than between RELS and REHS. The heatmap showed that LEHS increased abundance of argininesuccinic acid, deoxyuridine monophosphate (dUMP), PEP and guanosine diphosphate (GDP), whereas it decreased the abundance of acetyl-CoA, AMP, inosine monophosphate (IMP), D-ribulose-5-P, xylulose-5-P, uridine diphosphate (UDP), G-1-P, F-6-P, G-6-P, UMP, 6-phosphogluconic acid and D-erythrose-4-phosphate compared with the LELS group. Moreover, high starch:fat ratio increased abundance of UMP, IMP and AMP in regular energy diets, whereas it decreased the abundance of nicotinamide adenine dinucleotide (NAD) in regular energy diets.

3.9. Gene and protein abundances of AMPK pathway

Relative gene expression of the AMPK pathway is shown in Table 9 where a significant interaction was observed for *AMPKα1* ($P = 0.029$). In detail, the transition from low to high starch:fat ratio diet significantly up-regulated ileal gene expression of *AMPKα1* in birds offered low energy diets, but numerically decreased in regular energy diets. The energy reduction significantly decreased relative gene expression of *AMPKα1* in the ileum as a main effect ($P = 0.001$). In addition, energy reduction significantly down-regulated relative gene expression of *PGC1α*, *ACC*, *ESRRα* and *NRF1* in the ileum ($P < 0.05$) irrespective of starch:fat ratio.

Further analysis on relative protein abundance of the AMPK pathway is shown in Fig. 4. No significant interaction was observed for the protein abundance of AMPKα ($P = 0.061$), p-AMPKα:AMPKα ($P = 0.137$) and PGC1α ($P = 0.142$). As main effects, low energy decreased relative protein abundance of p-AMPKα:AMPKα in the ileum ($P = 0.013$), while high starch:fat ratio increased relative protein abundance of p-AMPKα:AMPKα ($P = 0.026$) and PGC1α ($P = 0.002$) in the ileum.

4. Discussion

The gastrointestinal tract (GIT) plays a pivotal role in partitioning of energy intake between heat energy loss and ATP production.

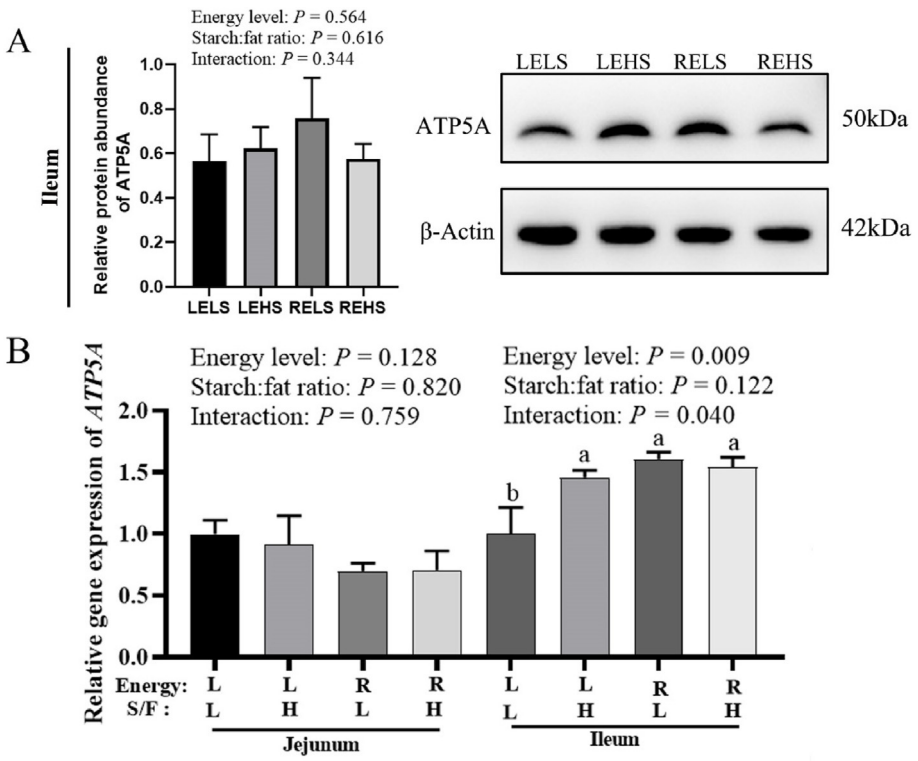

Fig. 1. Relative protein abundance of ATP5A in ileum (A) and gene expression of *ATP5A* in jejunum and ileum (B) of layer pullets at 14 weeks of age. Values in histogram are presented as mean ± SEM, $n = 6$ (for protein expression) or $n = 8$ (for gene expression). LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6–10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch:fat ratio diet (20:1). ATP5A = adenosine 5′-triphosphate synthase α subunit; L = low; R = regular; H = high; S/F = starch:fat ratio. [a,b] Values with different lowercase letters are different ($P < 0.05$).

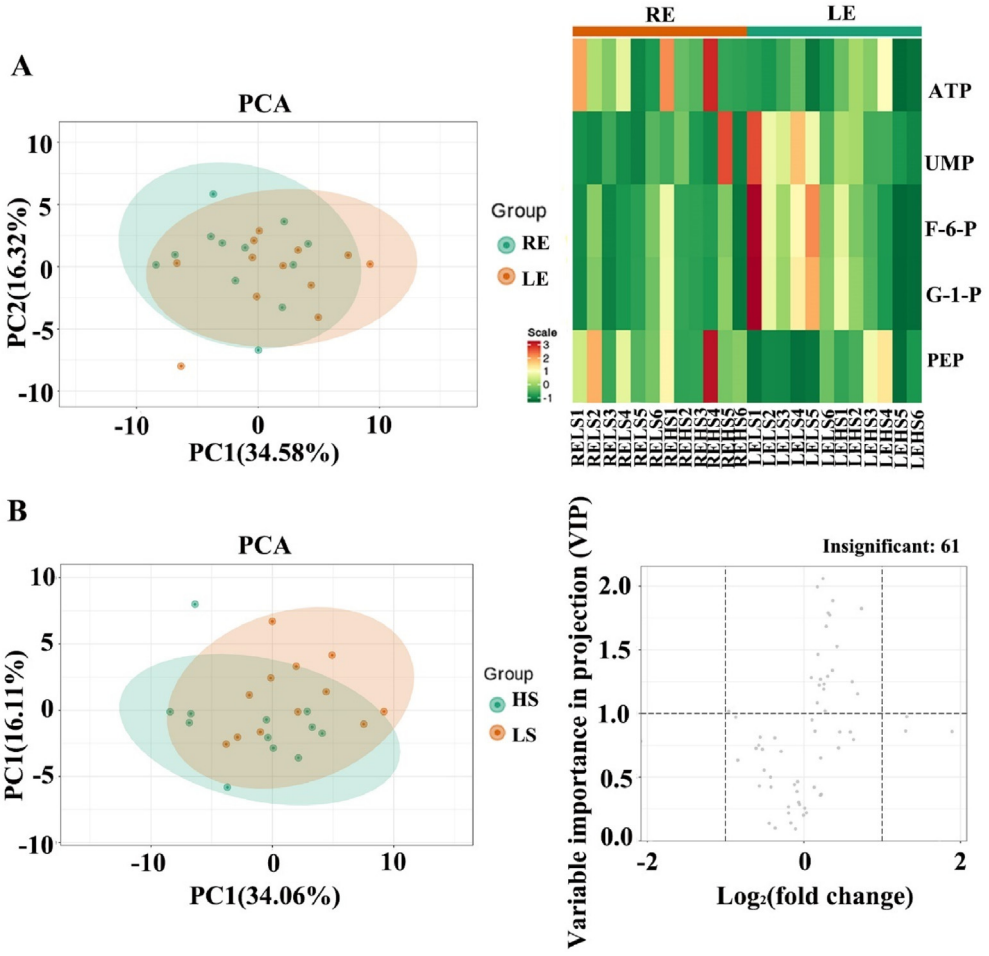

Fig. 2. Dietary treatment effects on metabolomic profile related to energy metabolism in ileal mucosa of layer pullets at 14 weeks of age. (A) Principal component analysis (PCA) and heatmap of metabolites between different energy levels. (B) PCA and volcano plot of metabolites between different starch:fat ratios. LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6–10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch:fat ratio diet (20:1), $n = 6$. PC = principal component; ATP = adenosine triphosphate; UMP = uridine monophosphate; F-6-P = fructose-6-phosphate; G-1-P = glucose-1-phosphate; PEP = phosphoenolpyruvate.

However, the GIT comprises only 4% to 6% of whole body weight in poultry (Shynkaruk et al., 2019; Rattanawut et al., 2021). Our study observed a numerical decrease in duodenal length and an increase in the weight of both the glandular stomach and gizzard following energy reduction. The dietary energy supply was positively correlated with GIT mass of animals, which was partially consistent with our findings (McLeod and Baldwin, 2000; Ahiwe et al., 2022). Specifically, the heavier stomach weight may be due to compensatory growth to increase the digestive surface and make up for energy deficiency (Taylor et al., 2021; Nilaweera and Speakman, 2018). Another aspect to consider is that low energy diets with high wheat bran inclusion result in relatively high crude fibre (CF) content, especially in the absence of corn starch. Indigestable fibre in the structure of wheat bran may slow down feed passage rate, increasing both the weight and function of the gizzard (Tuzun et al., 2021). In addition to gastrointestinal mass, dietary low energy also induced changes in intestinal morphology of broilers (Papadopoulos et al., 2018) and laying hens (Liu et al., 2024). In our study, although villus length did not differ between treatments, low energy diets significantly increased crypt depth of the jejunum. One explanation is that high fibre content in low energy diets potentially results in inflammation of intestinal epithelium due to harmful compounds produced by microbial fermentation (Kocer et al., 2021), which promotes the cell renewal rate and increases crypt depth (Rijke and van Dongen, 1980). This indicates that birds fed low energy diets with large crypt depth require extra energy for new cell production and that the utilization of nutrients is indirectly impaired (Wang et al., 2021). In contrast with our study, the reduction of villus length occurred in broilers (Ceylan et al., 2023) and laying hens (Kang et al., 2018) by reducing dietary energy levels. Variations in breed, growth stage and energy levels could explain the conflicting results. Moreover, the LELS group exhibited lower VCR compared with the LEHS and RELS groups. These findings are partly inconsistent with previous research, which indicated that oil inclusion could improve intestinal histomorphology (Thanabalan and Kiarie, 2022), and this difference may be attributed to variations in the rearing environment, as well as the type and amount of fat inclusion. In agreement with our studies, Adebowale et al. (2019) suggested that a low dietary starch:fat ratio, but not in a relatively high energy diet, could impair the intestinal villus growth in piglets. The underlying reason could be that the intestine is highly adaptable in starch digestion and absorption (Suvarna et al., 2005), especially when feeding low energy diets, which could potentially enhance the energy supply and promote intestinal growth. Although the inclusion of wheat bran differs in low and high starch:fat ratio diets, studies have shown that the inclusion of 4% of a fiber source may not affect GIT development (Guzman et al., 2016). These results led us to further

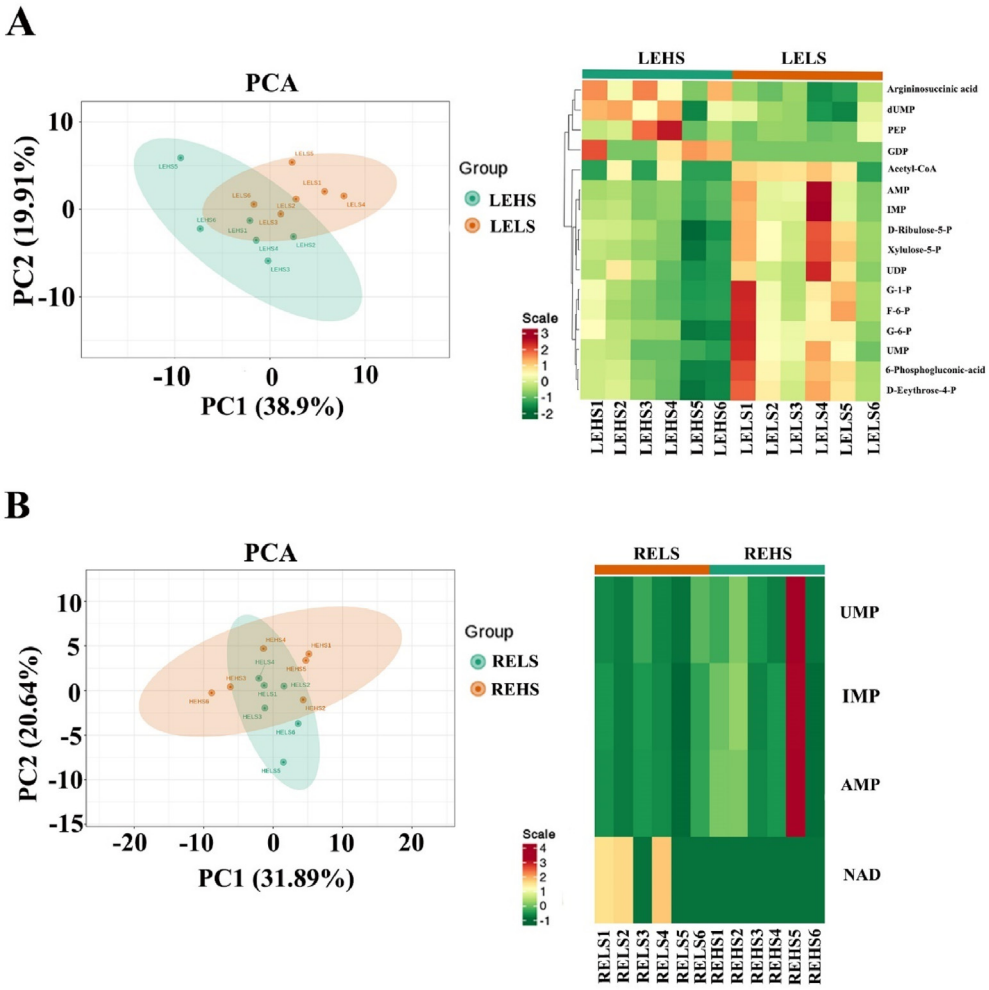

Fig. 3. Dietary starch:fat ratios differently affect metabolomic profile in ileal mucosa of layer pullets with low or regular energy feeding. (A) Principal component analysis (PCA) and heatmap of energy metabolites in the ileal mucosa of birds fed low energy diets. (B) PCA and heatmap of energy metabolites in the ileal mucosa of birds fed regular energy diets. LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6–10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch: fat ratio diet (20:1), n = 6. PC = principal component; dUMP = deoxyuridine monophosphate; PEP = phosphoenolpyruvate; GDP = guanosine diphosphate; AMP = adenosine monophosphate; IMP = inosine monophosphate; UDP = uridine diphosphate; G-1-P = glucose-1-phosphate; F-6-P = fructose-6-phosphate; G-6-P = glucose-6-phosphate; UMP = uridine monophosphate; NAD = nicotinamide adenine dinucleotide.

Table 9
Treatment effects on relative gene expression of the AMPK signaling pathway in the ileum of layer pullets at 14 weeks of age.[1]

Item	*AMPKα1*	*AMPKα2*	*PGC1α*	*ACC*	*ESRRα*	*NRF1*	*NRF2*	*TFAM*
Treatment								
LELS	1.00^{c}	1.00	1.00	1.00	1.00	1.00	1.00	1.00
LEHS	1.32^{b}	0.71	1.03	0.69	1.33	1.04	0.36	1.15
RELS	1.62^{a}	1.14	1.68	1.63	1.68	1.51	0.62	1.16
REHS	1.48^{ab}	1.07	1.83	1.90	1.81	1.76	0.22	1.13
Pooled SEM	0.063	0.116	0.111	0.124	0.116	0.078	0.178	0.033
Energy level effect								
LE	1.16^{b}	0.86	1.02^{b}	0.85^{b}	1.17^{b}	1.02^{b}	0.68	1.07
RE	1.55^{a}	1.11	1.75^{a}	1.77^{a}	1.74^{a}	1.64^{a}	0.42	1.14
Starch:fat ratio effect								
LS	1.31	1.07	1.34	1.32	1.34	1.26	0.81	1.08
HS	1.40	0.89	1.43	1.30	1.57	1.40	0.29	1.14
***P*-value**								
Energy level	0.001	0.297	<0.001	<0.001	<0.001	<0.001	0.475	0.304
Starch:fat ratio	0.405	0.459	0.622	0.924	0.282	0.205	0.159	0.365
E × S[2]	0.029	0.653	0.744	0.132	0.639	0.346	0.745	0.182

AMPK = adenosine monophosphate-activated protein kinase; *PGC1α* = peroxisome proliferator activated-receptor gamma coactivator 1α; *ACC* = acetyl CoA carboxylase; *ESRRα* = estrogen-related receptor α; *NRF* = nuclear respiratory factor; *TFAM* = transcription factor A, mitochondrial.
[a-c]Mean values within a column with different superscript letters are significantly different (P < 0.05), n = 8.
[1] LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6-10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch:fat ratio diet (20:1).
[2] E × S = the interaction between energy level and starch:fat ratio.

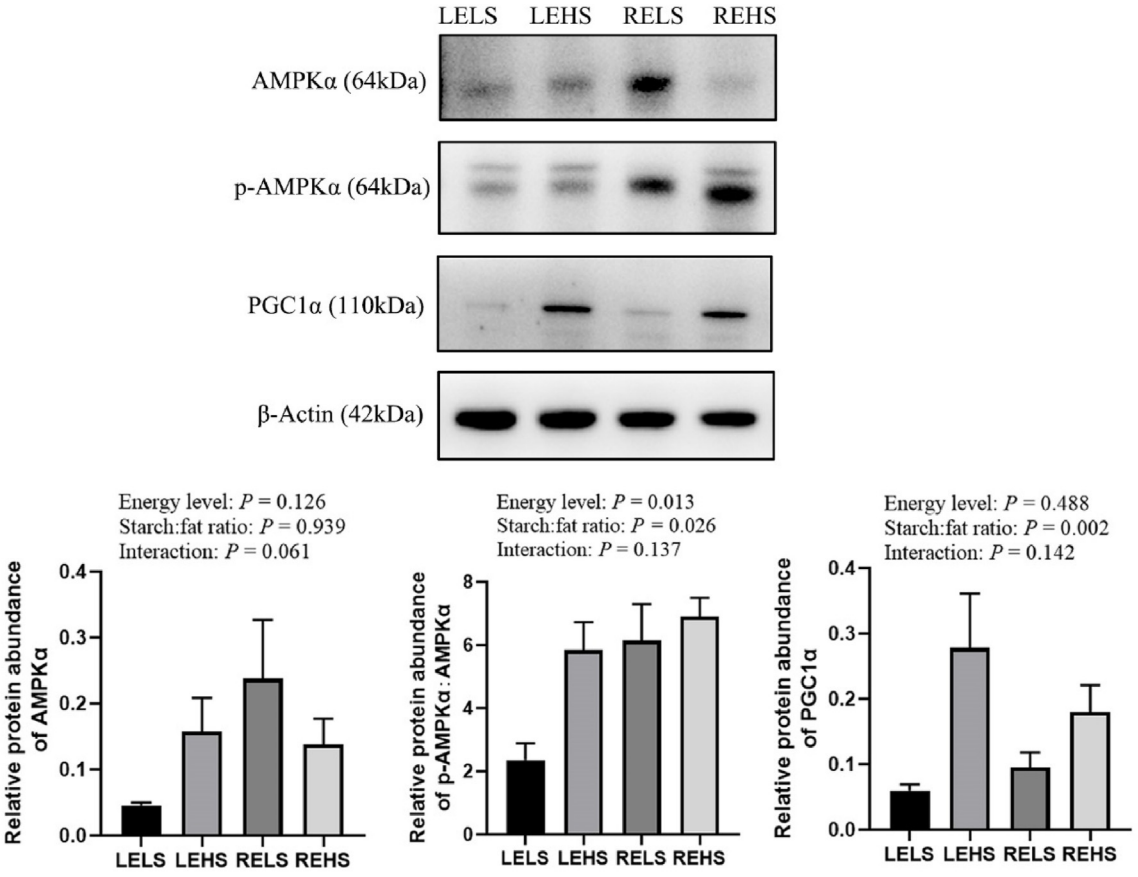

Fig. 4. Relative protein abundances of AMPKα, p-AMPKα:AMPKα and PGC1α in the ileum of layer pullets at 14 weeks of age. LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6–10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch:fat ratio diet (20:1). AMPK = adenosine monophosphate-activated protein kinase; PGC1α = peroxisome proliferator activated-receptor gamma coactivator 1α. Values in histogram are presented as mean ± SEM, $n = 6$.

explore the mechanism of dietary starch:fat ratios in regulating energy metabolism in the small intestine of birds fed low or regular energy diets.

After amino acids, glucose and fatty acids are absorbed into enterocytes; they can be metabolized and oxidized through different pathways, eventually entering the TCA cycle to yield energy. Malate is an essential metabolite in the TCA cycle, and the elevation of malate in birds fed a high starch:fat ratio diet indicates promotion of the TCA cycle (Arnold et al., 2022). Meanwhile, regular energy diets potentially facilitate the TCA cycle via improving the content of isocitrate and PEP in the ileal mucosa. The concentrations of energy substrates are closely correlated with activities of key enzymes in the TCA cycle including α-KGDH, IDH3 and CS (Madej et al., 2002; Pi et al., 2014). As expected, regular energy diets increased ileal gene expression of *α-KGDH*, *IDH3* and *CS*, which possibly facilitated the TCA cycle and ATP production (Zhang et al., 2019). Among these, IDH3 catalyzes the oxidative decarboxylation of isocitrate to produce α-ketoglutarate (α-KG) and nicotinamide adenine dinucleotide (NADH) (Charitou et al., 2015). Interestingly, the heatmap showed that the content of NAD was elevated by reducing starch:fat ratio in regular energy diets. The interplay of NAD and NADH, linking the TCA cycle and oxidative phosphorylation, plays an important role in energy homeostasis (Navas and Carnero, 2021). Electrons from NADH-linked energy substrates such as malate can enter the electron transport chain. The electron flow is accompanied by the transfer of protons from the matrix to the inner membrane space. This process creates a proton-motive force, thereby driving ATP synthase to produce considerable amounts of ATP (Ojano-Dirain et al., 2004). In our study, the gene expression of *ATP5A*, a subunit of ATP synthase, was improved by increasing starch:fat ratio in low energy diets. This could potentially promote efficient electron transport and facilitate ATP production (Zhou et al., 2021). Thus, high starch:fat ratio did not demonstrate beneficial effects on enzymes in the TCA cycle, but restored NADH-linked substrate (malate) and increased the gene expression of ATP synthase in the ileum of birds fed low energy diets.

Amino acids such as glutamine, glutamate and aspartate are the main energy substrates for the intestine (Wang et al., 2022; Wu, 2013). We measured free amino acid contents in the ileal mucosa, assuming that dietary treatments could regulate energy metabolism partly by reshaping amino acid profile. In our study, the contents of amino acids were not influenced by dietary energy levels except for Asn. In contrast, emerging evidence has shown that dietary energy levels predominantly affect the amino acid biosynthesis pathway in growing pigs (Fu et al., 2023). Variations in breed, energy levels and rearing environment could explain the differences. Different nutritional components of ingredients can interact with each other in the small intestine for absorption by enterocytes (Saberi et al., 2006). Our study showed that corn starch supplementation (high starch:fat ratio) increased the content of Asp, Glu, Gln, Ala and Thr in the ileal mucosa irrespective of energy levels. This is not without precedent; dietary starch supplementation influences the digestibility of amino acids and regulates amino

acid metabolism, and the degree to which this occurs depends on the proportion of amylopectin and amylose in starch (Yin et al., 2010). These amino acids mentioned above could be converted into substrates of the TCA cycle (Wu, 2013). For example, Glu and Gln are energy substrates for the synthesis of α-KG, an intermediate of the TCA cycle (He et al., 2022). Furthermore, Asp could be catalyzed by aminotransferase to form oxaloacetate (Encarnação et al., 2006; Wang et al., 2015). Ala and Thr are precursors of pyruvate that could subsequently convert into acetyl-CoA and are involved in the first step of this cycle (Encarnação et al., 2006). Thus, the increase in amino acids in a high starch:fat ratio diet can replenish the TCA cycle, manifested as higher malate levels mentioned above. However, the interpretation of amino acid profile in relation to energy metabolism is not sufficient because the digestibility of amino acids was not evaluated.

Energy metabolism is a complex set of processes for generating energy from nutrients, comprising a series of pathways such as glycolysis, the TCA cycle and adenine nucleotide metabolism. A volcano plot revealed that there were no statistically differential metabolites between low and high starch:fat ratios, of which a total of 61 metabolites were analyzed. Interestingly, there were differential metabolites between LELS and LEHS, RELS and REHS. This indicates that the energy metabolism in the ileum changed between different energy levels, and the pattern of metabolism had different responses to starch:fat ratios. Interestingly, energy reduction increased metabolites of glycolysis (F-6-P and G-1-P) but decreased concentrations of PEP and ATP in the ileal mucosa. Low energy feeding possibly causes metabolic abnormalities in animals (Fu et al., 2023). ATP is typically considered the main source of intracellular energy, while AMP to ATP and ADP to AMP ratios reflect intracellular energy status (Pi et al., 2014). High AMP to ATP ratio could activate the AMPK pathway and elevate ATP content, either by accelerating the ATP-production process, or by depressing ATP-consuming catabolic pathways (Hardie and Hawley, 2001). In our study, dietary energy reduction led to an accumulation of AMP but inactivated the AMPK signaling pathway, accompanied by lower ATP and a higher AMP to ATP ratio in the ileal mucosa. The results were inconsistent with a previous study by Ojano-Dirain et al. (2004), who believed that energy restriction decreased the energy demand for intestinal maintenance, resulting in accumulation of ATP. Compared with the single nucleotide scale, energy charge within the adenyl pool could be seen as a better approach for determining the energy status of the intestine (Hou et al., 2011). Although the content of TAN was increased, the AEC content which is indicative of high energy phosphate content was decreased in the ileal mucosa of birds fed low energy diets (Guo et al., 2017). Therefore, low energy diets increased the ratio of AMP to ATP and decreased AEC, altering the energy status in the ileal mucosa of layer pullets. Notably, this low energy status could be reversed by high starch:fat ratio, manifested as lower AMP, AMP to ATP, ADP to ATP and higher AEC in the LEHS group. Thus, it is reasonable to speculate that a high starch:fat ratio could stimulate the AMPK pathway in the ileum of birds fed low energy diets.

AMPK is a master regulator of cellular energy metabolism in the small intestine. The AMPK pathway could be activated by low energy status, accompanied by higher ATP content through promoting glucose and lipid breakdown (Franssen et al., 2021). Nevertheless, in our study, low energy inactivated the AMPK pathway in the ileum (Fig. 5). Consistent with this, dietary energy reduction resulted in low energy balance and depressed AMPK phosphorylation (Miao et al., 2017). Meanwhile, the up-regulated gene expression of *ACC* in birds fed with regular energy diets potentially promoted the synthesis of long-chain fatty acids (Chen et al., 2020; Hu et al., 2020). Another important finding is that high starch:fat ratio increased the ileal gene expression of *AMPKα* in birds fed with a low energy diet, but decreased it in a regular energy diet. In low energy conditions, starch could effectively provide energy and alleviate deficiencies by activating the AMPK signaling

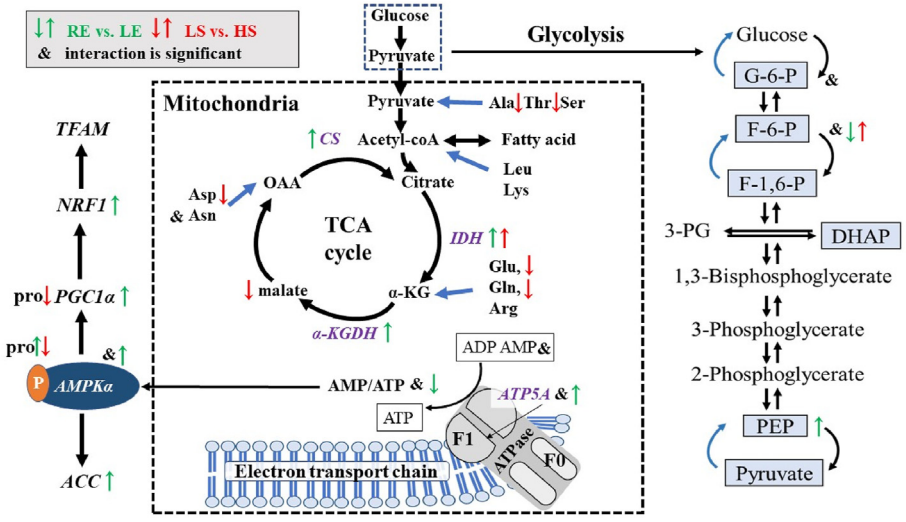

Fig. 5. Schematic representations of energy metabolites and enzymes involved in glycolysis, TCA cycle, ETC and AMPK pathway in ileum of layer pullets. The arrows (↑, increase; ↓, decrease) in green color represent the significant changes in ileum of birds fed regular energy diets compared with low energy diets (RE vs. LE). The arrows (↑, increase; ↓, decrease) in red color represent the significant changes in ileum of birds fed low starch:fat ratio diets compared with high starch:fat ratio diets (LS vs. HS). "&" represents a significant interaction between energy level and starch:fat ratio ($P < 0.05$). "pro" represents significant changes in protein abundance. LE = low energy diet (0.55 MJ/kg lower than that of regular energy diet), RE = regular energy diet (11.85 and 11.68 MJ/kg for birds from 6–10 weeks of age and 11-14 weeks of age, respectively), LS = low starch:fat ratio diet (10:1), HS = high starch:fat ratio diet (20:1). α-KGDH = α-ketoglutarate dehydrogenase complex; IDH = isocitrate dehydrogenase (NAD (+)) catalytic; CS = citrate synthase; AMPK = adenosine monophosphate-activated protein kinase; PGC1α = peroxisome proliferator activated-receptor gamma coactivator 1α; NRF = nuclear respiratory factor; TFAM = transcription factor A, mitochondrial; ATP5A = adenosine 5′-triphosphate synthase α subunit; OAA = oxaloacetate; TCA = tricarboxylic acid; α-KG = α-ketoglutarate; ADP = adenosine diphosphate; AMP = adenosine monophosphate; ATP = adenosine triphosphate; G-6-P = glucose-6-phosphate; F-6-P = frucose-6-phosphate; F-1,6-P = fructose-1,6-bisphosphate; 3-PG = 3-phosphoglycerate; PEP = phosphoenolpyruvate; DHAP = dihydroxyacetone phosphate; ETC = electron transport chain; Ala = alanine; Thr = threonine; Ser = serine; Leu = leucine; Lys = lysine; Asp = aspartate; Asn = asparagine; Glu = glutamine; Gln = glutamine; Arg = arginine.

pathway. This is not without precedent, as Liu et al. (2020) observed that starch supplementation could activate the AMPK signaling pathway and accelerate energy production. Also, dietary soybean oil inclusion has been shown to down-regulate the phosphorylation of the AMPK signaling pathway in piglets (He et al., 2024). Corn starch contains a large amount of resistant starch, a key nutritional component involved in AMPKα-regulated energy metabolism through microbial fermentation (Hu et al., 2010; Shang et al., 2017). Specifically, the energy demand of the intestine may be satisfied by feeding a regular energy diet, thereby reducing the efficacy of starch on energy regulation. As expected, AMPKα improved the downstream protein of PGC1α and estrogen-related receptor α (ESRRα). The AMPK-PGC1α pathway has been well documented to regulate mitochondrial metabolism (Zhang et al., 2021; Wang et al., 2019). PGC1α coactivates nuclear respiratory factor 1 (NRF1), while the latter can control the transcription and duplication of mitochondrial DNA (Sun et al., 2022). In addition to the functions mentioned above, the AMPK signalling pathway has been demonstrated to up-regulate intestinal nutrient absorption (Wu et al., 2022) and maintain the integrity of the intestinal barrier (Deng et al., 2018; Hayes et al., 2021). Therefore, diets with a high starch:fat ratio stimulate AMPKα phosphorylation and downstream protein under low energy status, potentially enhancing the intestinal function of layer pullets.

The BW and flock uniformity of all birds almost met the commercial standards (790 and 1120 g of BW at 10 and 14 weeks of age, respectively; 85% of flock uniformity), suggesting consistent initiation of egg production and strong laying persistency regardless of treatment effects. Energy reduction compromised the FCR in layer pullets and increased BW at 10 weeks of age. The decrease in intestinal energy metabolism did not directly impair body weight or flock uniformity in layer pullets fed the LELS diet. Considering this, further research should be conducted to confirm the benefits of high starch:fat ratio diets under low energy conditions. On the one hand, the long-term effects of dietary high starch:fat ratio on flock uniformity and laying performance of laying hens should be monitored. On the other hand, further study is warranted to explore whether dietary starch supplementation could improve energy status and thus ameliorate intestinal damage in layer pullets under pathogen challenge conditions.

5. Conclusion

Low energy feeding (0.55 MJ/kg lower than regular energy recommended by commercial standard) may not be sufficient for maintaining intestinal energy homeostasis of layer pullets. Supplementing corn starch at a dietary starch:fat ratio of 20:1 effectively stimulated the AMPK signaling pathway, increased amino acid levels, and boosted ATP production, thus counteracting the low energy status in the ileum. Therefore, the potential benefit of dietary low energy could be achieved by changing energy composition. The development of dietary energy levels and starch:fat ratio targeting intestinal energy metabolism may be an alternative approach to improve intestinal function.

CRediT authorship contribution statement

Qiuyu Jiang: Conceptualization, Formal analysis, Methodology, Writing – original draft, Data curation. **Lihua Zhao:** Writing – review & editing, Investigation. **Jiaqi Lei:** Data curation, Formal analysis, Methodology. **Xiangfei Geng:** Investigation, Methodology. **Xiang Zhong:** Conceptualization, Supervision. **Bingkun Zhang:** Funding acquisition, Methodology, Supervision, Writing – review & editing, Conceptualization.

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.

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