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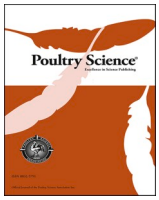

Full-Length Article

Transcriptomic analysis of ileal adaptations and growth responses in growing hens supplemented with alanyl-glutamine dipeptide

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ABSTRACT

The growing phase of laying hens is crucial for growth and development due to its direct impact on their productivity during laying phase. During initial growth phase, intestinal tract undergoes rapid development which requires plenty of nutrients to help laying hens grow and mature. This study investigated the effect of Alanyl-Glutamine (**Aln-Gln**) levels on growth performance, ileal morphology and transcriptomic analysis of growing *Hy-line* brown hens. A total of 480 day old *Hy-line* brown chicks having similar body weight (**BW**) were randomly divided to be fed diets having 0%, 0.1%, 0.2% and 0.3% Aln-Gln for 6-wks (8 replicates/group, 15 birds/replicate). One bird from every pen was slaughtered and morphological parameters of ileum were evaluated. Results taken on day 42 revealed an improved average daily gain (**ADG**), final body weight (**FBW**) and feed-to-gain ratio (**F/G**) in the birds that consumed 0.2% and 0.3% Aln-Gln supplemented diet ($P < 0.05$). Ileal morphological assays showed that villus height, villus width and villus to crypts ratio (**V/C**) were significantly increased at 42 days of age in birds fed diets with 0.2% Aln-Gln ($P<0.05$). The RNA sequencing (**RNA-Seq**) was executed to identify differentially expressed genes (**DEGs**) among groups that found 2265 DEGs (1256 up-regulated; 1009 down-regulated) in ileum tissue. According to the Kyoto Encyclopedia of Genes (**KEGG**) and Genomic Pathway Enrichment Analysis, majority of DEGs indicated change in metabolic pathways. Genes related to growth factors, intestinal morphology and protein metabolism were up-regulated in test groups as compared to control group. In conclusion, addition of Aln-Gln to the diet improved growth performance and ileum development in growing hens; transcriptomic analysis revealed up-regulation of genes related to growth and intestinal morphology.

Introduction

Poultry growth, production, enzymatic activity, and immunity primarily rely on proteins. The building blocks of these proteins are amino acids (**AA**), that are essential components of eggs and poultry (Beski et al., 2015). To maximize the efficiency of poultry production, it's important to maintain a balanced and appropriate intake of AA. Over-consumption of AA can negatively impact poultry growth and production (Réhault-Godbert et al., 2019). During the growing period, the health and development of hens are vital factors that influence their laying performance (Lang et al., 2019). The size, weight, and overall health of growing hens have a direct impact on egg production and livability, by directly affecting the cost-effectiveness of poultry businesses. Providing proper nutrition and care during the growing period is essential to ensure success in poultry farming (He et al., 2021).

Glutamine (**Gln**) is a precursor during synthesis of arginine and proline, the constituents of body proteins. Glutamine upregulates the expression of genes necessary for cell growth and downregulates the oxidative stress and immunity-related genes (Hanczakowska and Niwińska, 2013). Glutamine is considered as a key amino acid in the feed of growing hens. The intrinsic Gln of the bird's body is not enough for disease and stressed conditions so it's necessary to be given in the feed. The utilization of free Gln in diets is not efficient (Xue et al., 2018). Thus, Aln-Gln dipeptide is suggested to be an alternative to Gln monomers due to its increased stability and solubility compared to free Gln. (Zhu et al., 2020). It plays a significant role in accelerating the growth of

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intestinal villi and improving epithelium structure where nutrients are absorbed, thus improving growth performance and feed efficiency in hens. The effect of glutamine on intestinal microflora also improves animal health. The digestive intestinal tract is the main site of glutamine metabolism (Bortoluzzi et al., 2018). This progression leads to the synthesis of pyrimidine and purine nucleotides, and also antioxidant glutathione (Cruzat et al., 2018).

The progress in RNA-Seq has opened up more possibilities for research by allowing the gathering of extensive data about the transcriptome at the gene level (Wu et al., 2024). Currently, majority of RNA-Seq studies in poultry nutrition are primarily focused on disease resistance and growth performance (Dar et al., 2022), meat parameters (Park et al., 2017), and feed efficiency (Karimi et al., 2022), with limited studies on ileum morphology in growing *Hy-line* brown hens. Hence, we consider this methodology applicable for revealing the gene expression profile and pathway of ileum development in growing *Hy-line* brown hens (Zhu et al., 2023). In this study, RNA-Seq technology was used to study transcriptomic profiles of ileal tissues. Bio-informatics analysis and validation experiments were used to find DEGs that regulate growth performance and ileum development in growing *Hy-line* brown hens. This study aims to provide a theoretical basis for regulating Aln-Gln to increase growth performance and ileum development in *Hy-line* brown hens through transcriptomic analysis and a finding that could significantly impact the poultry industry.

Material Methods

The handling of birds and protocols in the current experiment was performed in accordance with the YZU Animal Care and Use Committee of Yangzhou University, Jiangsu, China (under permission number SYXK [Su] 2021-0020).

Experimental design, birds, and diets

One-day-old 480 healthy, *Hy-line* brown chicks were arbitrarily divided into 4 experimental groups with eight replicates of 15 chicks each in a 42-day trial. All chicks were kept in cages (37 × 30 × 40 cm) that were facilitated with external access devices for water and feed throughout the trail and raised according to the Management guide of *Hy-line* brown hen (Hy-Line, 2023). The test diets were formulated using the soya-corn basal diets supplemented with 0% (control), 0.1%, 0.2%, and 0.3 % Aln-Glu (groups A, B, C and D). The diets were formulated as shown (Table 1) to meet the nutritional requirements of chicks according to recommendations of NRC, 1994. A powder form of Aln-Gln (Shandong Chen-long Pharmaceutical Co., Ltd, China) was added to the diets. On the first day, the initial body weight (**IBW**) of each bird was measured. At the end of each week, the fasted birds were weighed, and their ADFI, ADG, and F/G were also measured.

Table 1
Composition of diet (0-6 weeks).

Ingredients (%)	A	Addition of Aln-Gln Powder		
		B	C	D
Corn	66.10	66.00	66.00	65.9
Soybean	29.00	29.00	28.90	28.99
Wheat Bran	0.200	0.200	0.200	0.200
Aln-Gln Powder	-	0.1	0.2	0.3
Met	0.20	0.20	0.20	0.20
Lys	0.20	0.20	0.20	0.20
Salt	0.30	0.30	0.30	0.30
Limestone	1.30	1.30	1.30	1.30
CaHPO3	1.70	1.70	1.70	1.70
Premix	1	1	1	1
Total	100	100	100	100
ME (MJ/Kg)	11.98	11.98	11.98	11.98
CP %	18.4	18.4	18.4	18.4
Lys Dig. %	1.126	1.126	1.126	1.126
Met Dig. %	0.47	0.47	0.47	0.47
Thr Dig. %	0.68	0.68	0.68	0.68
Trp Dig. %	0.20	0.20	0.20	0.20
Arg Dig. %	1.22	1.22	1.22	1.22
Ile Dig. %	0.74	0.74	0.74	0.74
Val Dig. %	0.84	0.84	0.84	0.84
CF %	2.78	2.78	2.78	2.78
Ca %	1.078	1.078	1.078	1.078
P Av. %	0.45	0.45	0.45	0.45
Na %	0.023	0.023	0.023	0.023
Cl %	0.041	0.041	0.041	0.041

Per kilogram of diet, the following are provided: 11,000 IU of vitamin A, 3,000 IU of vitamin D3, 20 IU of vitamin E, 3 mg of vitamin K3 (as menadione), 0.02 mg of vitamin B12, 6.5 mg of riboflavin, 10 mg of calcium pantothenate, 40.1 mg of niacin, 0.2 mg of biotin, 2.2 mg of thiamine, 4.5 mg of pyridoxine, 1,000 mg of choline, 125 mg of ethoxyquin (antioxidant) Additionally, per kilogram of diet, the following are provided: 66 mg of Mn (as manganese oxide), 70 mg of Zn (as zinc oxide), 80 mg of Fe (as ferrous sulfate), 10 mg of Cu (as copper sulfate), 0.3 mg of Na (as sodium selenite), 0.4 mg of I (as calcium iodate), 0.67 mg of iodized salt

A group having 0% Aln-Gln and also as the control group; B group having 0.1% Aln-Gln; C group having 0.2% Aln-Gln; D group having 0.3% Aln-Gln

Tissue sample collection

On 42 days, one bird with an average body weight from each replicate was taken and butchered with a knife method following the permitted protocols of the YZU Animal Care Advisory Committee. The ileum part from the small intestine was isolated and sampled. It was washed and fixed in a 4% solution of para-formaldehyde for ileal histological analysis. The ileal mucosal samples were collected by rubbing the microscopic slide on the ileal segments and stored at -80˚C for transcriptomic analysis. The process of pullet selection and ileal sampling is shown in Fig. 1.

Ileal morphological analysis

The ileal segments were fixed in paraffin. The 5-μm thick tissue sections were subjected to deparaffinization, rehydration, and staining with hematoxylin and eosin. A minimum of three films per fragment and three segments from each bird were acquired for analysis. After the collection of ileal mucosal samples, the villus height (VH), villus width (VW), and crypt depth (CD) were measured using an NIKON YS100 light microscope. The average measurements were then used to calculate the villi-crypt ratio (VCR).

RNA Extraction, library construction, and sequencing

Total RNA was isolated using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's guidelines. The quality of the RNA was evaluated with an Agilent 2100 Bio-analyzer (Agilent Technologies, Palo Alto, CA, USA) and confirmed via RNase-free agarose gel electrophoresis. Subsequently, eukaryotic mRNA was enriched using Oligo(dT) beads. For prokaryotic mRNA, rRNA was removed using the Ribo-ZeroTM Magnetic Kit (Epicentre, Madison, WI, USA). The enriched mRNA was then fragmented using a fragmentation buffer and reverse transcribed into cDNA using the NEB Next Ultra RNA Library Prep Kit for Illumina (NEB #7530, New England Bio labs, Ipswich, MA, USA). The resulting double-stranded cDNA fragments were subjected to end-repair, A base addition, and ligation to Illumina sequencing adapters. The ligation reaction was purified using AMPure XP Beads(1.0X) and subsequently underwent polymerase chain reaction (PCR) amplification. The resulting cDNA library was sequenced using Illumina Novaseq6000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

Differential Expression Analysis and Enrichment

The expression level of every gene was determined using the fragments per kilobase per million reads (FPKM) method to identify the

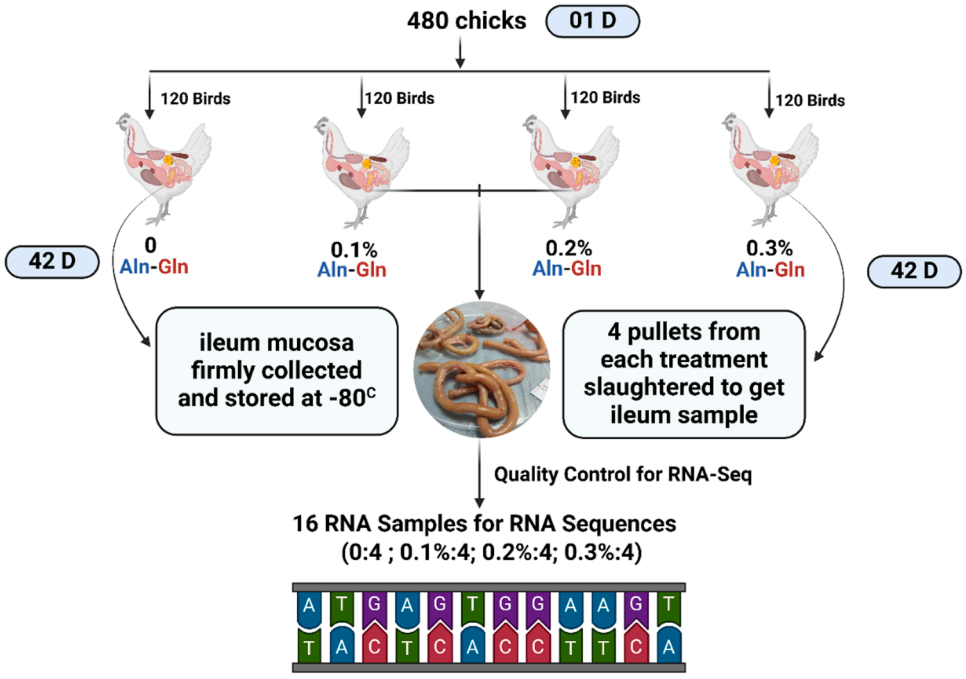

Fig. 1. This diagram depicts the experimental design for RNA-Seq.

differentially expressed genes among the 4 groups. Gene abundance was assessed using RSEM (http://deweylab.biostat.wisc.edu/rsem/). DESeq2 was used for the differential expression analysis, and genes with |log2(fold change) |≥0.585 and P value<0.05 were considered significantly differentially expressed. Additionally, functional enrichment analysis, including Gene Ontology (GO, http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/), was conducted at P≤0.05 to identify DEGs significantly enriched in GO terms and metabolic pathways compared to the entire transcriptome background. The GO functional enrichment and KEGG pathway analyses were performed using Goa tools (https://github.com/tanghaibao/Goatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home).

Gene Expression Analysis by qRT-PCR

To confirm the consistency and precision of the RNA Seq gene expression data, we conducted quantitative real-time PCR (qRT-PCR) validation of differentially expressed genes (DEGs) in several biological

Table 2
Primer sequences for the genes utilized in the qRT-PCR.

Name	Details	Gene ID	Product length	Primer	TM
ND4L	NADH de-hydrogenase subunit-4L	63549492	121	F: AAACCCCATCATTCGCCCTT	60.97
				R: TTGTGTAGGTGGTCAGAGCC	59.09
ND4	NADH de-hydrogenase subunit-4	63549470	292	F: ACCTACCTGCCTCCTGAACA	59.89
				R: AGGTCTGTTTGGCGTAAGCA	60.06
IGF1	Insulin-like growth factor 1	418090	197	F: TGGCCTGTGTTTGCTTACCTT	60.25
				R: CACAACTCTGGAAGCAGCAC	60.01
OCLN	occludin	396026	196	F: GCGGTTACTACTACAGCCCC	59.01
				R: TAGCGCCAGATCTTACTGCG	60.67
CLDN1	claudin 1	424910	200	F: ACCCACAGCCTAAGTGCTTC	59.96
				R: AGGTCTCATAAGGCCCCACT	59.02
ANPEP	alanyl aminopeptidase, membrane	395667	164	F: GTCACCCAACGCTTCAACAC	59.97
				R: TGCACGACCTCCTTGTTCTC	60.10
ADA	adenosine deaminase	419194	278	F: ACCCCTCTTGTTAGGCTTGC	60.11
				R: AAGGATGGGGGAGTCGATGA	59.76
TXNDC12	thioredoxin domain containing 12	772208	236	F: GCCCAGGAGAAGCTAACAGG	60.21
				R: GCCAGGCAGAGGAGATAACA	59.81
PCK1	phosphoenolpyruvate carboxykinase 1	396458	86	F: GAAGGCTTAGGTGTGGACCC	59.96
				R: CCTTGGGGCAGGCATTTAGA	60.25
CPT1A	carnitine palmitoyltransferase 1A	423118	114	F: ATCCAGTCCCTCTCAGCCAT	59.97
				R: CCATTAAGCGATGGCAACGG	60.04
PSAT1	phosphoserine aminotransferase 1	427263	219	F: TCAGAAGCTTGCGACGTTCA	60.75
				R: CAAGCCCTTGCAAGATTGGG	59.75
PDE6B	phosphodiesterase 6B	395092	176	F: CATGCCGATCGCTGTAGTCT	59.97
				R: TTGGTTTGCGCAACATAGCC	60.04
ATP6V0A4	ATPase H+ transporting V0 subunit a4	418104	216	F: GAGCTCAGCAGGAACAACCT	59.96
				R: GTGTGGTCTTCCTCACCCAG	60.04
B3GAT1	beta-1,3-glucuronyltransferase 1	373949	247	F: CTGGGGAACTGATGGTCCAC	60.04
				R: CAAAGCAGCTGTTCACCCAC	59.93
PLIN1	perilipin 1	415487	177	F: GACCACAGCAAGGTACACGA	59.97
				R: GATTGCTGCTGGGAGACCTT	60.03

samples from each group. By using TRI-zol, we isolated RNA from ileum tissues and determined the concentration of the isolated RNA using NanoDrop2000 (Thermo Fisher Scientific). The Prime Script-RT reagent kit with the gDNA Eraser was used to generate a reverse transcription of 1mg RNA. The qRT-PCR amplification was done using TB Green Premix Ex Taq II (Tli RNaseHPlus) on a Bio-Rad CFX384 real-time PCR system, following the protocol described in Luo et al., (2019). The gene expression levels were calculated using the $2^{\Delta\Delta}$Ct technique, with GAPDH serving as the control, as described by Livak and Schmittgen, (2001). The primer sequences for genes used in the qRT-PCR is shown in Table 2. All primers were designed by AZENTA Life Sciences Co., Ltd., located in Massachusetts, United States.

Statistical Analysis

The data of all parameters were analyzed using one-way analysis of variance (ANOVA), and the general linear model procedure was used in SPSS17.0; LSD's multiple test was used for multiple comparisons. The statistical significance was considered at $P \leq 0.05$ for all the analyses. The figures were created using Graph-Pad Prism 8 (Graph Pad Software Inc., San Diego, CA).

Results

Sequencing Data and Quality Control

After conducting transcriptomic analysis of ileum tissue samples from 6-week-old *Hy-line* brown hens, quality control generated 70.34 Gb of clean reads. Each sample produced clean reads of more than 30.60 GB, and the percentage of Q30 bases was more than 94.18%. The GC content ranged from 46.46% to 49.67%. More than 92.55% of the reads were successfully mapped to the genome of the laying hens, spanning various regions of the chicken reference genome, predominantly within the coding sequences of exons and introns. The total fragments of the coding regions ranged from 84.73% to 86.74%, with the number of introns ranging from 8.99% to 10.79%, and the percentage of intergenic regions ranging from 3.90% to 5.04%. These results confirmed the reliability of the data for further analysis (Table 3).

Gene Expression Profile and Differential Expression Analysis

Gene expression levels were typically measured by FPKM or transcripts per million reads (TPM). The FPKM method can eliminate the effect of gene length and sequencing volume differences on the calculation of the gene expression and was therefore selected to perform the expression analysis. The overall distribution of expression levels is shown in Fig. 2. Principal components analysis (PCA) was performed and the results are shown in Fig. 2. We obtained 69.5% for PC1 and 16.7% for PC2. Based on this RNA sequencing data analysis of ileum tissue from the 6-week-old brown hen pullets, a total of 17,571 expressed genes were detected and subjected to differential expression analysis to further identify transcriptional changes between ileum tissue of pullets having different levels of Aln-Gln in diets. A total of 2265 DEGs were identified by comparing the gene expression between four treatments ($|\log2(\text{fold change})| \geq 1.5$, P-value < 0.05), with 1256 up-regulated and 1009 down-regulated genes. The volcano plot showed statistically significant differences in gene expression levels between different treatments Fig. 3.

GO and KEGG Enrichment Analysis of DEGs

The GO is a database created by the Gene Ontology Consortium. It enables the categorization of genes in the selected gene set: the biological process involved, components that make up the cell, and molecular functions achieved, among others. For this, we employed GO analysis to annotate RNA-Sequence functionally. A total of 44 GO terms were identified by comparing 4 different groups. The top 20 GO terms for DEGs in the comparative library in the ileum are listed in Fig. 2. These GO terms include cellular processes, biological regulation, metabolic process, binding, catalytic activity, transporter activity, membrane part, organelles, and cell part. Significant enrichment of biological processes with GO terms related to growth, protein metabolism, synthesis, and translocation is shown in Fig. 2. The gene associated with these GO terms may be essential for the synthesis and metabolism of AA, which is required for chick ileum's growth and intestinal development.

To better understand the modulatory role of DEGs in metabolism, we performed KEGG enrichment analysis. The total DEGs were condensed into 243 KEGG pathways, and the top 20 KEGG pathways are shown in Fig. 2. In ileum tissue, DEGs showed significant enrichment in metabolic pathways, the PPAR signaling pathway, lipid atherosclerosis, cysteine and methionine metabolism, and the chemokine signaling pathway. Analyzing the genes connected to the pathway can give insights into the temporal and spatial expression of associated genes with AA metabolism in the ileum.

Table 3
Gene sequencing data of *Hy-line* brown chicks (0-6 weeks).

Sample	Raw-Reads	Raw-bases	Clean-reads	Clean-bases	Total mapped	Q30 (%)	GC content(%)
A1-Aln-Gln	38271306	5740695900	38021570	5642914332	35299830 (93.35%)	94.57	47.34
A2-Aln-Gln	48231938	7234790700	47866016	7087881255	44760881 (93.84%)	94.76	46.52
A3-Aln-Gln	46223572	6933535800	45972838	6830372797	42386335 (93.16%)	94.18	48.65
A4-Aln-Gln	47945182	7191777300	47641892	7072861253	44556378 (93.86%)	94.38	46.89
B1-Aln-Gln	40847150	6127072500	40592026	6020012312	37377076 (92.55%)	95.33	49.67
B2-Aln-Gln	40460184	6069027600	40241946	5961331647	37525033 (93.57%)	95.59	47.34
B3-Aln-Gln	42768594	6415289100	42543926	6315837758	39832018 (93.93%)	95.59	46.88
B4-Aln-Gln	37104466	5565669900	36886158	5472667186	34489432 (93.85%)	95.39	46.89
C1-Aln-Gln	48601216	7290182400	48265474	7182102691	44987098 (93.67%)	95.49	47.49
C2-Aln-Gln	52529796	7879469400	52254910	7753668439	48892390 (93.88%)	95.74	47.14
C3-Aln-Gln	58361910	8754286500	57997660	8607143449	54276569 (94.01%)	94.74	46.83
C4-Aln-Gln	45531608	6829741200	45250216	6705512548	42129028 (93.48%)	94.79	47.38
D1-Aln-Gln	41228030	6184204500	40992338	6092668907	38321241 (93.94%)	95.04	46.46
D2-Aln-Gln	42243864	6336579600	41977636	6230226486	38987916 (93.32%)	94.88	48.52
D3-Aln-Gln	36941974	5541296100	36675252	5438938861	34296869 (93.93%)	94.43	46.81
D4-Aln-Gln	40100498	6015074700	39868188	5899916850	37300464 (93.84%)	94.93	46.54

Note: Raw reads: total number of entries of raw sequencing data; Raw bases: total amount of raw sequencing data; Clean reads: total number of entries of post-quality control sequencing data; Clean bases: total amount of post-quality control sequencing data; Total mapped: the number of clean reads that can be localized to the genome; Q30 (%): quality assessment of post-quality control sequencing data, referring to the percentage of bases with sequencing quality above 99.9% of the total bases; Guanine-cytosine (GC) content (%): the sum of G and C bases corresponding to quality control (QC) data as a percentage of the total bases.
A group having 0% Aln-Glu and also as the control group; B group having 0.1% Aln-Glu; C group having 0.2% Aln-Glu; D group having 0.3% Aln-Glu

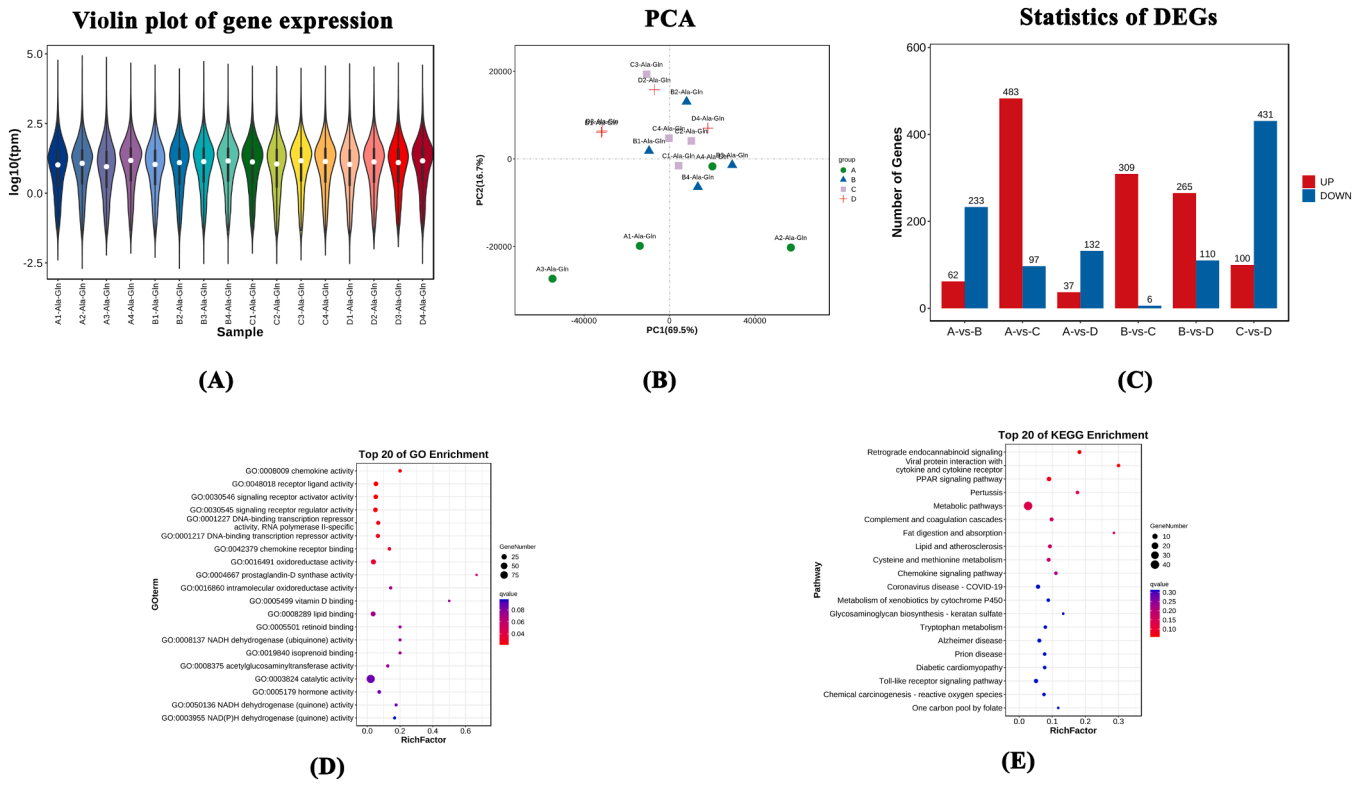

Fig. 2. (A) Violin plot of gene expression; (B) Principal component Analysis (PCA); (C) Statistics of DEGs; (D) The top 20 gene ontology (GO) terms of DEGs in the relative library of ileal tissue; (E) Top 20 KEGG analysis of DEGs between groups.

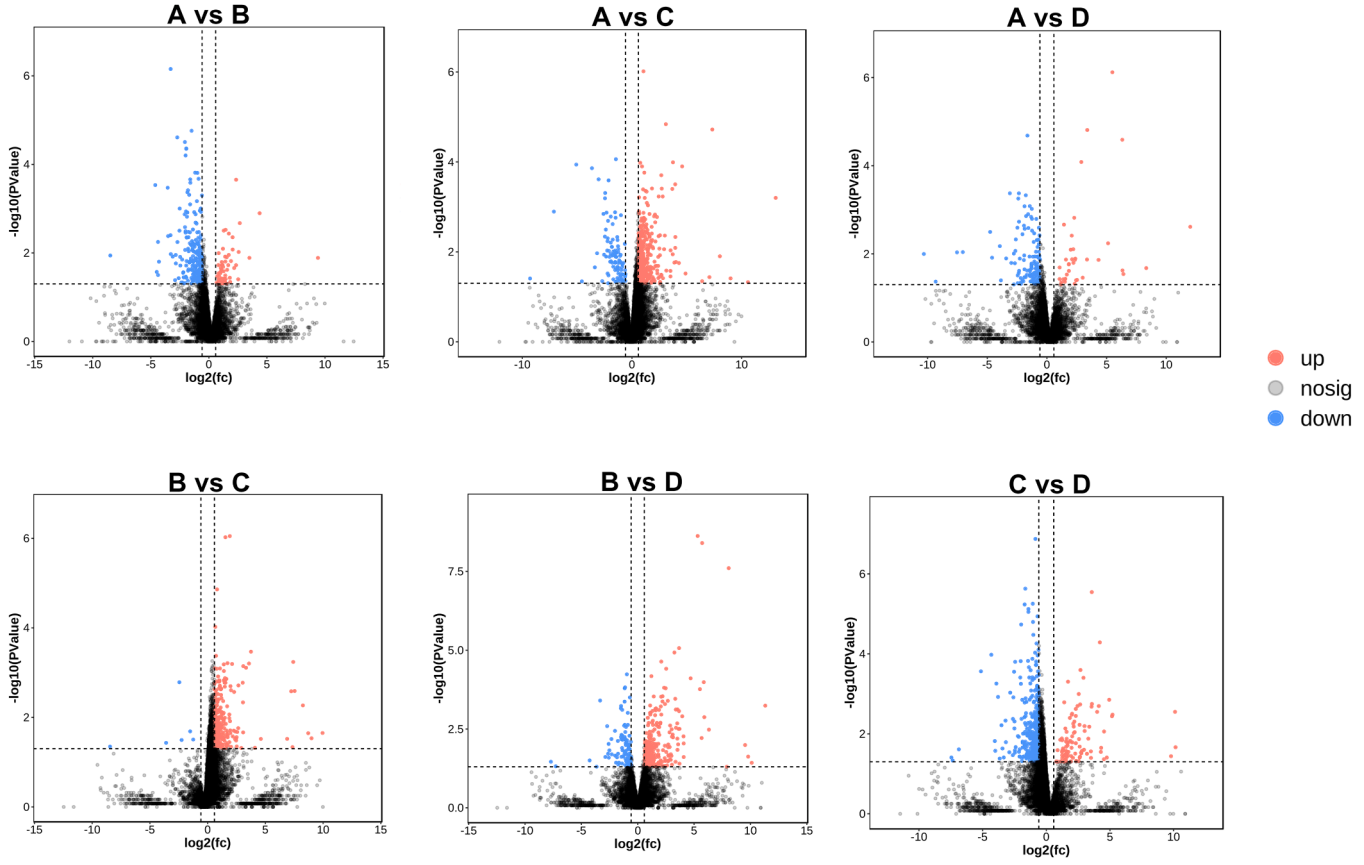

Fig. 3. Comparison of DEGs between groups by Volcano plots.

Validation of DEGs by qRT-PCR

According to the RNA-expression profile, we identified 15 DEGs (10 upregulated and 5 downregulated genes) selected for qRT-PCR, which possessed the characteristics of abundant expression and higher FC. These genes were Insulin-like growth factor 1 (**IGF1**), NADH dehydrogenase subunit 4L (**ND4L**), NADH dehydrogenase subunit 4 (**ND4**), claudin 1 (**CLDN1**), occluding (**OCLN**), alanyl aminopeptidase, membrane (**ANPEP**), adenosine deaminase (**ADA**), thioredoxin domain containing 12 (**TXNDC12**), phosphoenolpyruvate carboxykinase 1 (**PCK1**), carnitine palmitoyltransferase 1A (**CPT1A**), phosphoserine aminotransferase 1 (**PSAT1**), phosphodiesterase 6B (**PDE6B**), ATPase H+ transporting V0 subunit a4 (**ATP6V0A4**), beta-1,3-glucuronyltransferase 1 (**B3GAT1**), and perilipin 1 (**PLIN1**). As shown in Fig. 4, the relative expression of IGF1, ND4L, ND4, CLDN1, OCLN, ANPEP, ADA, TXDNC12, PCK1, and CPT1A were significantly increased ($P \leq 0.05$) in those groups who fed Aln-Gln as compared to control group. Whereas the expression levels of PSAT1, PDE6B, ATP6V0A4, B3GAT1, and PLIN1 decreased ($P \leq 0.05$) in the test groups compared to the control group. These findings align with the variation trend observed in the RNA-Seq data, thus indicating the reliability and accuracy of our sequencing data. Moreover, it highlights that RNA-Seq is an excellent reference method for expression profiling.

Growth Performance

As revealed in Table 4, the Intake of Aln-Gln had a significant effect on ADG, F/G, and FBW. By increasing Aln-Gln in the feed significantly increased ADG and FBW ($P < 0.05$), and F/G improved linearly with increasing levels of Aln-Gln in diet ($P < 0.05$). The ADG and FBW were significantly higher in the group C and D having 0.2% Aln-Gln and 0.3% Aln-Gln than in the 0.1% Aln-Gln group ($P < 0.05$), and 0.2% to 0.3% Aln-Gln was associated with lower F/G and the most uniform body weight at 6-wk old versus the control group (0% Aln-Gln).

Ileal morphology

The effect of different levels of Aln-Gln on the ileal morphology of a 42-day old *Hy-line* brown hen was shown in Table 5. The VH in the ileum was higher in groups B and C than the other two groups. The VW in the ileum was higher in groups B and C than the control group ($P < 0.05$), But crypt depth was decreased in Aln-Gln groups as compared to the control group. The villi-to-crypts ratio was significantly higher in group C and the B and D groups than the control group ($P < 0.05$).

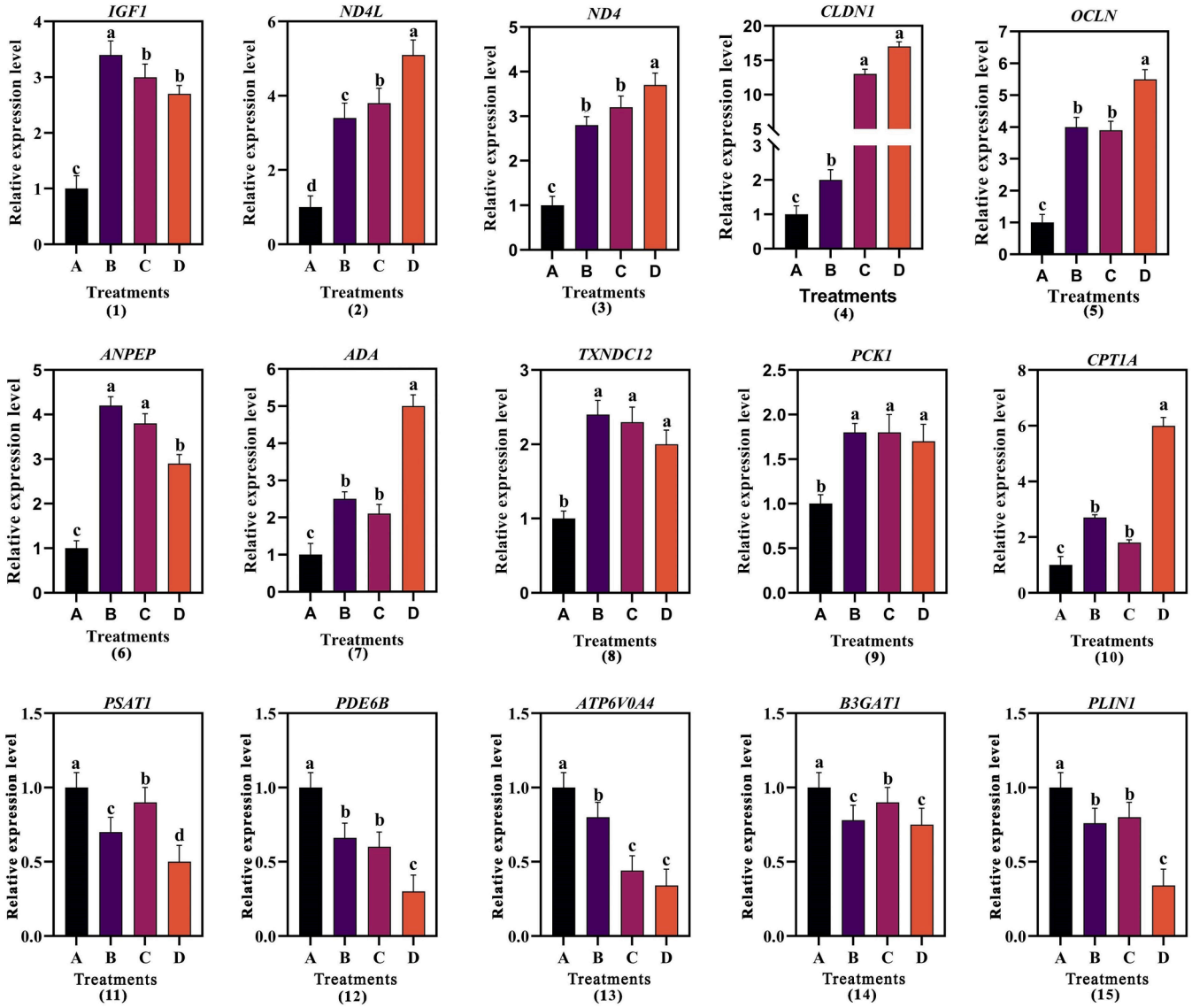

Fig. 4. Validation of RNA-seq results by qRT-PCR, presented as mean ± SEM.

Table 4
Growth Performance of *Hy-line* brown chicks (0-6 weeks).

Items	A	B	C	D	SEM	*P-value*
IBW (g)	38.76	38.66	38.90	38.74	0.046	0.10
ADFI (g)	27.10	27.27	27.30	27.43	0.031	1.73
ADG (g)	9.73^{c}	10.54^{b}	11.41^{a}	11.31^{a}	0.122	0.00
F/G	2.78^{a}	2.58^{b}	2.39^{c}	2.42^{c}	0.028	0.00
FBW (g)	447.54^{c}	481.43^{b}	518.48^{a}	514.58^{a}	5.154	0.01

Note: Raw reads: total number of entries of raw sequencing data; Raw bases: total amount of raw sequencing data; Clean reads: total number of entries of post-quality control sequencing data; Clean bases: total amount of post-quality control sequencing data; Total mapped: the number of clean reads that can be localized to the genome; Q30 (%): quality assessment of post-quality control sequencing data, referring to the percentage of bases with sequencing quality above 99.9% of the total bases; Guanine-cytosine (GC) content (%): the sum of G and C bases corresponding to quality control (QC) data as a percentage of the total bases.
A group having 0% Aln-Glu and also as the control group; B group having 0.1% Aln-Glu; C group having 0.2% Aln-Glu; D group having 0.3% Aln-Glu

Table 5
Ileum Morphology of *Hy-line* brown chicks (0-6 weeks).

Items	A	B	C	D	SEM	*P*-value
VH (μm)	592.51^{c}	629.39^{a}	627.42^{a}	602.49^{b}	4.08	0.00
VW (μm)	87.81^{c}	93.48^{a}	94.20^{a}	90.14^{b}	0.71	0.01
CD (μm)	116.25^{a}	104.52^{b}	102.73^{b}	107.90^{b}	1.65	0.04
VCR	5.09^{c}	6.02^{b}	6.11^{a}	5.58^{b}	0.24	0.02

a, b, c, showed in the same row with different letters differ significantly at $P<0.05$.
A group having 0% Aln-Gln and also as the control group; B group having 0.1% Aln-Gln; C group having 0.2% Aln-Gln; D group having 0.3% Aln-Gln
VH= villus height, VW= villus width, CD= crypt depth and VCR= villi-to-crypts ratio

Discussion

The growing period of laying hens is a crucial phase that significantly impacts their growth, development, and production performance after they start laying eggs. Therefore, it is important to ensure that hens receive proper and balanced nutrients during this phase.

The intestine plays a vital role in chicks' digestion and absorption of nutrients and considered as a key component of their immune system. However, during the initial stages of bird growth, the development of the small intestine is incomplete and delicate. As a result, growth often results in common issues such as functional disorders and structural damage to the gut (Rehman et al., 2007).

Alanyl-glutamine, a stable dipeptide of Gln, has been observed to have better blood Gln availability than the free form (Minguetti-Câmara et al., 2014). A number of studies have demonstrated that Aln-Gln can enhance animal performance and intestinal integrity in-vitro and in animal models (Ueno et al., 2011; Zhou et al., 2012; Rodrigues et al., 2013). This study aimed to assess whether dietary supplementation with Aln-Gln could improve growth performance, intestinal development, and transcriptomic analysis performance using growing hens as the animal model.

In this study, supplementation with Aln-Gln at levels of 0.1% and 0.2% in the diet improved the performance and ileum morphology of growing *Hy-line* Brown birds. Previous reports have provided inconsistent results regarding the effects of Gln supplementation on bird performance. Sakamoto et al. (2006) observed no difference in performance between 14-day-old broilers fed corn-soy diets with or without 0.1% Gln supplementation, whereas Bartell and Batal, (2007) noted a significant improvement in body weight gain with the same level of Gln supplementation. However, the effects of Gln are likely to be more pronounced in the presence of stressors, as suggested by Novak et al. (2002), who proposed that Gln may be conditionally essential for broiler health and productivity under critical conditions. Researchers found that Gln significantly improved growth performance in growing birds (Dai et al., 2012; Olubodun et al., 2015; Hu et al., 2016).

As the current study revealed Aln-Gln enhanced ileum's villus height, V:C ratio, and decreased crypt depth. These findings are consistent with previous research that demonstrated the pronounced effects of dietary Gln supplementation on small intestinal development. Additionally, it has been estimated that the small intestinal mucosa catabolizes all of the dietary glutamine and most of the glutamate and aspartate (Sakamoto et al., 2006). The studies revealed that L-Glutamine might offer enterocytes metabolic fuel to benefit gut morphology and mucosa (Bartell and Batal, 2007). Increased villus height may result in a stronger absorptive potential for available nutrients. Low crypt depth suggests lowering metabolic cost for intestinal epithelium turnover (Xue et al., 2018), which may be reflected by the lower F/G that was observed in the current study. This might be because the crypt serves as the villus factory, and more crypts depth suggest quicker tissue turnover for villus renewal as required in reaction to inflammation caused by infections or associated toxins (Namroud et al., 2017). A higher V:C ratio, thus, points to better performance, less gastrointestinal secretion, and more absorption of nutrients. Moreover, greater weight increase and lower FCR with adding Aln-Gln may be the proof that Gln has a favorable effect on enterocytes to prevent injury, as shown by less crypt depths and longer villus height on d 42 (Jazideh et al., 2014). An alternate theory holds that Gln enhances stress or challenge recovery without increasing enterocyte renewal rate (Tan et al., 2017). This demonstrates how broiler chicks that had starved for 48 hours after hatching recovered from delayed small intestine growth with a 0.1% Gln supplementation (Namroud et al., 2017). A variety of studies revealed genes related to production and ileum development discovered in meat type birds, including Myf5, MyoG (Genxi et al., 2014), and IGF-2 (Beccavin et al., 2001). However, limited studies have investigated ileum development and growth in laying hens during the growing period from a transcriptomic perspective. In this experiment, RNA-Seq was conducted on laying hens of growing phase to uncover differentially expressed genes (DEGs) associated with intestinal development, metabolism, and growth. The total 2265 DEGs were identified by comparing gene expression in 6-week-old growing laying hens. The GO enrichment analysis revealed that the DEGs, consisting of downregulated and upregulated genes, were associated with various GO biological process terms. These GO biological processes were primarily involved in biological processes, cellular components, and molecular functions. Based on the ileum's mucosal transcriptomic profile in the current experiment, AA metabolism is likely an important factor affecting birds' intestinal development and growth (Han et al., 2023).

In addition, our screening of DEGs identified several genes linked to growth, intestinal morphology, and protein metabolism, Such as ND4L, ND4, IGF1, OCLN, CLDN1, ANPEP, ADA, TXNDC12, PCK1, CPT1A, PSAT1, PDE6B, ATP6V0A4, B3GAT1, and PLIN1. The most important upregulated gene expressed in the test groups was IGF-1, which plays a key role in the growth of different tissues, such as muscle cells. There are different genetic variations that affect how the gene is expressed during transcription and translation. These variations could impact the growth and development of animals (Karabag and Ersal, 2019). The same theory was observed in the current study, and a significant improvement was found in the growth performance of growing birds. We found genes *ND4L, ND4*, and *ATP6V0A4* of oxidative phosphorylation. The *ND4L* and ND4 were upregulated, and the upregulated genes of this pathway appear in major mitochondrial complexes, including complex I, III, IV, and ATP synthase (Rich and Maréchal, 2010). These complexes form the electron transport chain, which helps in energy production in mitochondria, a basic need for the metabolism and growth of birds in the growing phase (Liu et al., 2020). We found significantly higher expression of *CLDN1* and *OCLN* genes in the test groups, especially in the C and D groups. The role of *CLDN1* and *OCLN* was observed to maintain the integrity of the gut barrier in bovine colostrum, which is the source of

essential nutrients and maternal antibodies for newborn animals (Stewart et al., 2016). It leads to increased *CLDN1* and *OCLN* expressions in the ileal tissues of bovine and chicken (Stephens and Johnson, 2017). Our results revealed that ileal morphology was significantly improved upon up-regulation of these genes. The *ANPEP* gene was also upregulated in groups B and C compared to the control group. In small intestine, aminopeptidase N plays a vital role in the digestion of peptides that are formed through the hydrolysis of protein by pancreatic and gastric proteases (Volf et al., 2021). The *ADA* gene was upregulated in test groups, which is responsible for an enzyme that plays a key role in participating in purine metabolism (Iwaki-Egawa et al., 2004). The *TXNDC12* gene up-regulated in the following experiment, which is responsible for a redox protein that reduces oxidative stress in animals (Zhu et al., 2024). The *PCK1* and *CPTIA* genes were upregulated in this study which act as a machinery for gluconeogenesis and lipid metabolism in animal bodies (Yu et al., 2021; Li et al., 2022) This study's downregulated *PSAT1* gene in growing hens indicates the biosynthesis of serine in magnum, which may be needed for synthesizing egg-white protein, as it showed expression in laying hens (Sah et al., 2021). All these genes were interconnected with metabolism to provide secondary support for improving the growth performance and ileal morphology of *Hy-line* brown hens, as indicated by the results of the current study. The present study showed downregulated *PDE6B* and *B3GAT1* genes, which are responsible for the production of hormones named melatonin and glycosaminoglycan biosynthesis (Morin et al., 2001; Roy et al., 2013). The *PLIN1* knockout in adipocytes increased basal lipolysis and decreased the size of lipid droplets; it resists diet-induced obesity in chickens. As in growing stage of laying hens we need to maintain body weight because over-weight may have difficulties in laying stage. The *PLIN1* gene was downregulated in test group because it showed no expression in early stages of life. It mostly needed to be upregulated to control the weight of the hens during the laying period (Sun et al., 2019). Taken together, these data reveal that Aln-Gln had a positive effect on growth performance and ileal morphology of growing hens as explained by relevant gene expression.

Conclusion

We have concluded that the addition of Aln-Gln to the diet improved growth performance and ileum development of *Hy-line* brown growing hens; transcriptomic analysis revealed upregulation of gene expression related to growth and intestinal morphology.

Disclosures

The authors declare no conflict of interest.

Availability of Data and Materials

The datasets supporting the conclusions of this article are included within the article.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Haiming Yang reports financial support was provided by Huai'an science and technology planning project. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.104479.

References

Bartell, S.M., Batal, A.B., 2007. The effect of supplemental glutamine on growth performance, development of the gastrointestinal tract, and humoral immune response of broilers. Poult. Sci. 86, 1940–1947.
Beccavin, C., Chevalier, B., Cogburn, L.A., Simon, J., Duclos, M.J., 2001. Insulin-like growth factors and body growth in chickens divergently selected for high or low growth rate. Journal of Endocrinology 168, 297–306.
Beski, S.S.M., Swick, R.A., Iji, P.A., 2015. Specialized protein products in broiler chicken nutrition: A review. Animal Nutrition 1, 47–53. https://doi.org/10.1016/j.aninu.2015.05.005. Available at.
Bortoluzzi, C., Rochell, S.J., Applegate, T.J., 2018. Threonine, arginine, and glutamine: Influences on intestinal physiology, immunology, and microbiology in broilers. Poult. Sci. 97, 937–945. https://doi.org/10.3382/ps/pex394. Available at.
Cruzat, V., Rogero, M.M., Keane, K.N., Curi, R., Newsholme, P., 2018. Glutamine: Metabolism and immune function, supplementation and clinical translation. Nutrients. 10, 1–31.
Dai, S.F., Gao, F., Xu, X.L., Zhang, W.H., Song, S.X., Zhou, G.H., 2012. Effects of dietary glutamine and gamma-aminobutyric acid on meat colour, pH, composition, and water-holding characteristic in broilers under cyclic heat stress. Br. Poult. Sci. 53, 471–481.
Dar, M.A., Ahmad, S.M., Bhat, B.A., Dar, T.A., ul Haq, Z., Wani, B.A., Shabir, N., Kashoo, Z.A., Shah, R.A., Ganai, N.A., Heidari, M., 2022. Comparative RNA-Seq analysis reveals insights in Salmonella disease resistance of chicken; and database development as resource for gene expression in poultry. Genomics 114, 110475. https://doi.org/10.1016/j.ygeno.2022.110475. Available at.
Genxi, Z., Ying, T., Tao, Z., Jinyu, W., Yongjuan, W., 2014. Expression profiles and association analysis with growth traits of the MyoG and Myf5 genes in the Jinghai yellow chicken. Mol. Biol. Rep. 41, 7331–7338.
Han, G.P., Kim, J.H., Kim, J.M., Kil, D.Y., 2023. Transcriptomic analysis of the liver in aged laying hens with different eggshell strength. Poult. Sci. 102, 102217. https://doi.org/10.1016/j.psj.2022.102217. Available at.
Hanczakowska, E., Niwińska, B., 2013. Glutamine as a feed supplement for piglets: A review. Annals of Animal Science 13, 5–15.
He, W., Li, P., Wu, G., 2021. Amino Acid Nutrition and Metabolism in Chickens. Adv. Exp. Med. Biol. 1285, 109–131.
Hu, H., Bai, X., Shah, A.A., Wen, A.Y., Hua, J.L., Che, C.Y., He, S.J., Jiang, J.P., Cai, Z.H., Dai, S.F., 2016. Dietary supplementation with glutamine and γ-aminobutyric acid improves growth performance and serum parameters in 22- to 35-day-old broilers exposed to hot environment. J. Anim. Physiol. Anim. Nutr. (Berl) 100, 361–370.
Hy-Line, 2023. Management Guide 2013. Management Guide.
Iwaki-Egawa, S., Namiki, C., Watanabe, Y., 2004. Adenosine deaminase 2 from chicken liver: Purification, characterization, and N-terminal amino acid sequence. Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology 137, 247–254.
Jazideh, F., Farhoomand, P., Daneshyar, M., Najafi, G., 2014. The effects of dietary glutamine supplementation on growth performance and intestinal morphology of broiler chickens reared under hot conditions. Turkish Journal of Veterinary and Animal Sciences 38, 264–270.
Karabag, K., Ersal, M., 2019. Insulin-Like Growth Factor-1 (IGF-1) in Poultry. In: INTERNATIONAL BIOLOGICAL, AGRICULTURAL AND LIFE SCIENCE CONGRESS 20.
Karimi, P., Bakhtiarizadeh, M.R., Salehi, A., Izadnia, H.R., 2022. Transcriptome analysis reveals the potential roles of long non-coding RNAs in feed efficiency of chicken. Sci. Rep. 12, 1–17. https://doi.org/10.1038/s41598-022-06528-6. Available at.
Lang, W., Hong, P., Li, R., Zhang, H., Huang, Y., Zheng, X., 2019. Growth performance and intestinal morphology of Hyline chickens fed diets with different diet particle sizes. J. Anim. Physiol. Anim. Nutr. (Berl) 103, 518–524.
Li, Z.Q., Li, J.J., Lin, Z.Z., Zhang, D.H., Zhang, G.F., Ran, J.S., Wang, Y., Yin, H.D., Liu, Y.P., 2022. Knockdown of CPT1A Induce Chicken Adipocyte Differentiation to Form Lipid Droplets. Revista Brasileira de Ciencia Avicola /Brazilian Journal of Poultry Science 24, 1–8.
Liu, H., Chen, Y., Zhu, X., Zhang, M., Li, W., 2020. Identification of Key Genes and Pathways Associated with Chronic Obstructive Pulmonary Disease Based on Bioinformatics Analysis. In: ACM International Conference Proceeding Series, pp. 1–18.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25, 402–408.
Luo, J.J., Zhang, Y., Sun, H., Wei, J.T., Khalil, M.M., Wang, Y.W., Dai, J.F., Zhang, N.Y., Qi, D.S., Sun, L.H., 2019. The response of glandular gastric transcriptome to T-2 toxin in chicks. Food and Chemical Toxicology 132, 110658. https://doi.org/10.1016/j.fct.2019.110658. Available at.
Minguetti-Câmara, V.C., de C. R. Marques, A., Schiavon, F.P.M., Vilela, V.R., Brusch, M. L., Bazotte, R.B., 2014. A comparison of the effects of oral glutamine dipeptide, glutamine, and alanine on blood amino acid availability in rats submitted to insulin-induced hypoglycemia. Nutrients. 6, 4520–4530.
Morin, F., Lugnier, C., Kameni, J., Voisin, P., 2001. Expression and role of phosphodiesterase 6 in the chicken pineal gland. J. Neurochem. 78, 88–99.

Namroud, N.F., Shivazad, M., Zaghari, M., Madadgar, O., Nourijelyani, K., 2017. Impact of dietary glutamine on amino acid digestibility values and intestinal morphometric parameters in neonate chicks. S. Afr. J. Anim. Sci. 47, 440–453.
Novak, F., Heyland, D.K., Avenell, A., Drover, J.W., Su, X., 2002. Glutamine supplementation in serious illness: A systematic review of the evidence. Crit. Care Med. 30, 2022–2029.
NRC. 1994. Nutrient Requirements of Poultry.
Olubodun, J.O., Zulkifli, I., Farjam, A.S., Hair-Bejo, M., Kasim, A., 2015. Glutamine and glutamic acid supplementation enhances performance of broiler chickens under the hot and humid tropical condition. Italian Journal of Animal Science 14, 25–29.
Park, W., Rengaraj, D., Kil, D.Y., Kim, H., Lee, H.K., Song, K.D., 2017. RNA-seq analysis of the kidneys of broiler chickens fed diets containing different concentrations of calcium. Sci. Rep. 7, 1–11. https://doi.org/10.1038/s41598-017-11379-7. Available at.
Réhault-Godbert, S., Guyot, N., Nys, Y., 2019. The golden egg: Nutritional value, bioactivities, and emerging benefits for human health. Nutrients. 11, 1–26.
Rehman, H.U., Vahjen, W., Awad, W.A., Zentek, J., 2007. Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. Arch. Anim. Nutr. 61, 319–335.
Rich, P.R., Maréchal, A., 2010. The mitochondrial respiratory chain. Essays Biochem. 47, 1–23.
Rodrigues, R.S., Oliveira, R.A.C., Li, Y., Zaja-Milatovic, S., Costa, L.B., Braga Neto, M.B., Kolling, G.L., Lima, A.A., Guerrant, R.L., Warren, C.A., 2013. Intestinal epithelial restitution after TcdB challenge and recovery from clostridium difficile infection in mice with alanyl-glutamine treatment. Journal of Infectious Diseases 207, 1505–1515.
Roy, P., Kumar, B., Shende, A., Singh, A., Meena, A., Ghosal, R., Ranganathan, M., Bandyopadhyay, A., 2013. A Genome-Wide Screen Indicates Correlation between Differentiation and Expression of Metabolism Related Genes. PLoS. One 8.
Sah, N., Kuehu, D.L., Khadka, V.S., Deng, Y., Jha, R., Wasti, S., Mishra, B., 2021. RNA sequencing-based analysis of the magnum tissues revealed the novel genes and biological pathways involved in the egg-white formation in the laying hen. BMC. Genomics. 22, 1–16.
Sakamoto, M.I., Murakami, A.E., Silveira, T.G.V., Fernandes, J.I.M., De Oliveira, C.A.L., 2006. Influence of glutamine and vitamin E on the performance and the immune responses of broiler chickens. Revista Brasileira de Ciencia Avicola /Brazilian Journal of Poultry Science 8, 243–249.
Stephens, C.S., Johnson, P.A., 2017. General and Comparative Endocrinology Occludin expression and regulation in small follicles of the layer and broiler breeder hen. General and Comparative Endocrinology. https://doi.org/10.1016/j.ygcen.2017.02.010. Available at.
Stewart, S., K. Robinson, and G. Zhang. 2016. CLDN1 and CLDN2 expression in chickens Tissue expression of claudin-1 and claudin-2 tight junction proteins in chickens 1 Sydney Stewart, Kelsy Robinson, and Guolong Zhang Department of Animal Science, Oklahoma State University, Stillwater, OK 74077. Department of Animal Science, Oklahoma State University, Stillwater, OK 74077:1–8.
Sun, Y., Li, R., Zhai, G., Zhang, X., Wang, Y., 2019. DNA methylation of the PLIN1 promoter downregulates expression in chicken lines. Arch. Anim. Breed. 62, 375–382.
Tan, B., Liu, H., He, G., Xiao, H., Xiao, D., Liu, Y., Wu, J., Fang, J., Yin, Y., 2017. Alanyl-glutamine but not glycyl-glutamine improved the proliferation of enterocytes as glutamine substitution in vitro. Amino Acids. 49, 2023–2031.
Ueno, P.M., Oriá, R.B., Maier, E.A., Guedes, M., de Azevedo, O.G., Wu, D., Willson, T., Hogan, S.P., Lima, A.A.M., Guerrant, R.L., Brent Polk, D., Denson, L.A., Moore, S.R., 2011. Alanyl-glutamine promotes intestinal epithelial cell homeostasis in vitro and in a murine model of weanling undernutrition. American Journal of Physiology - Gastrointestinal and Liver Physiology 301, 612–622.
Volf, J., Rajova, J., Babak, V., Seidlerova, Z., Rychlik, I., 2021. Detoxification, hydrogen sulphide metabolism and wound healing are the main functions that differentiate caecum protein expression from ileum of week-old chicken. Animals 11, 3155.
Wu, J., Wang, Y., An, Y., Tian, C., Wang, L., Liu, Z., Qi, D., 2024. Identification of genes related to growth and amino acid metabolism from the transcriptome profile of the liver of growing laying hens. Poult. Sci. 103.
Xue, G.D., Barekatain, R., Wu, S.B., Choct, M., Swick, R.A., 2018. Dietary L-glutamine supplementation improves growth performance, gut morphology, and serum biochemical indices of broiler chickens during necrotic enteritis challenge. Poult. Sci. 97, 1334–1341.
Yu, S., Meng, S., Xiang, M., Ma, H., 2021. Phosphoenolpyruvate carboxykinase in cell metabolism: Roles and mechanisms beyond gluconeogenesis. Mol. Metab. 53, 101257. https://doi.org/10.1016/j.molmet.2021.101257. Available at.
Zhou, Y., Zhang, P., Deng, G., Liu, X., Lu, D., 2012. Improvements of immune status, intestinal integrity and gain performance in the early-weaned calves parenterally supplemented with l-alanyl-l-glutamine dipeptide. Vet. Immunol. Immunopathol. 145, 134–142. https://doi.org/10.1016/j.vetimm.2011.10.020. Available at.
Zhu, Q., Cai, Y., Xiao, C., Kong, L., Pan, X., Song, B., Song, Z., 2023. RNA sequencing transcriptomics and metabolomics in three poultry breeds. Sci. Data 10, 1–6.
Zhu, Z., Z. Hu, S. Ojima, X. Yu, M. Sugiyama, and H. K. Ono. 2024. Critical Involvement of the Thioredoxin Reductase Gene (trxB) in Salmonella Gallinarum-Induced Systemic Infection in Chickens.
Zhu, J., Yang, W., Wang, B., Liu, Q., Liu, Q., Zhong, X., Zhong, X., Gao, Q., Liu, J., Liu, J., Huang, J., Lin, B., Lin, B., Tao, Y., Tao, Y., 2020. Metabolic engineering of Escherichia coli for efficient production of l-alanyl-l-glutamine. Microb. Cell Fact. 19, 1–10. https://doi.org/10.1186/s12934-020-01369-2. Available at.