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Investigating the Molecular Mechanisms of Lysine's Effect on Feed Intake in Chicks Through the Hypothalamus using RNA-seq and Metabolomics

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ABSTRACT

Background: There is a current lack of research on the molecular pathways by which lysine regulates feed intake in chicks. Regulation is controlled by the hypothalamus and lysine metabolism links the availability of amino acids and nutritional perception with food intake. The mechanism underlying this phenomenon remains unclear. To investigate the regulatory mechanism of lysine on feed intake in chicks, this study used Yao chickens as a research model. The levels of lysine in the feed of chicks were adjusted to determine the effect on feed intake. The hypothalamus tissue of the chicks was analyzed using RNA-seq and metabolomics to identify the relevant genes. We employed Gene Ontology and identification of signaling pathways to characterize the molecular regulatory biological pathways of lysine in poultry feeding.

Methods: This study used RNA-seq technology to investigate the genes and marker metabolites involved in lysine regulation of feed intake in chickens. The study explored the molecular mechanism by which dietary lysine regulates feed intake in chickens *via* the hypothalamus. Four hundred healthy 1-day-old chicks (Yao chickens) were randomly divided into four groups, with five replicates in each group (n = 20).

Result: There was a significant difference in feed intake among chicks fed with diets containing 0.65% (abbreviated as MGO), 0.85% (FIH), 1.0% (MGS) and 1.2% (FIL) lysine levels. The group with the highest feed intake was FIH, while the group with the lowest feed intake was FIL. By conducting RNA-seq of the hypothalamic tissue of chickens in both the highest and lowest feed intake groups, we obtained 2006 differentially expressed genes, comprising 1275 upregulated genes and 731 downregulated genes. Fourteen genes were related to food intake, including growth hormone, recombinant glutamate receptor, metabotropic 1 (GRM1) and recombinant glutamate receptor, metabotropic 3 (GRM3). Using liquid chromatograph mass spectrometer (LC-MS) metabolomics, we identified 25 differential metabolites. Nine metabolites, including $C_3H_5NO_4$, $C_{10}H_{15}NO_2$ and $C_{13}H_{14}N_20_3$, were downregulated, while 16 metabolites, including $C_2H_8NO_3P$, $C_3H_7NO_3$ and $C_7H_7NO_2$, were upregulated. Marker metabolites included 5-hydroxytryptophan and L-valine. A KEGG enrichment analysis revealed that the differential metabolites between the FIH and FIL groups were enriched in 26 pathways, with the significantly enriched pathway being the ABC transporter protein pathway (ID: map02010). RNA-seq and LC-MS analyses revealed that in chicks, the expression of the neuropeptides cocaine- and Amphetamine-Regulated Transcript Protein (CARTPT) and neuropeptide Y (NPY)/agouti-related protein (AGRP), along with genes such as sucrose synthase (SS 2), recombinant cholecystokinin A receptor (CCKAR) and recombinant cholecystokinin B receptor (CCKBR), was regulated by the hypothalamic feeding regulation center that senses the level of lysine in the feed. These molecules bind to specific receptors and initiate a signaling cascade in specific hypothalamic neuronal groups, thereby regulating chick feed intake and in turn affecting growth, development and production performance.

Key words: Chick, Food intake, Hypothalamus, Lysine, Metabolomics, RNA-seq.

INTRODUCTION

Lysine, also referred to as 2,6-diaminohexanoic acid, is an alkaline amino acid that typically exists in the form of lysine hydrochloride and lysine sulfate (Li Junlin *et al*., 2020). Lysine has several primary functions in poultry. Lysine is a critical component of protein synthesis involved in the formation of peptide hormones and is thus closely related to growth, making it a crucial member of the growth amino acids (Wang Xinxi *et al*., 2011). Lysine is also a precursor in the synthesis of carnitine that converts some unsaturated fatty acids into energy through â-oxidation in fat metabolism (Khan *et al*., 1979). Lysine also helps to maintain the acid-base balance in animals (Li Junlin *et al*., 2020) and it can enhance the appetite of poultry and

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promote the rapid growth of young animals (Chen Panpan, 2018). Research on lysine and its related applications has been conducted for more than a century (Jia Hongmin *et al*., 2020). Although lysine does not rank first among the limiting amino acids in corn and soybean meal diets, it nevertheless plays a crucial role in protein deposition in poultry by regulating the rates of protein synthesis and decomposition, thereby affecting the growth performance of chickens. It is thus an essential amino acid in chickens' nutritional requirements (Emmert *et al*., 1997; Cemin *et al*., 2017; Baker *et al*., 2002; Schutte *et al*., 1995; Dahariya *et al*., 2022). The appropriate concentration of lysine is crucial to the production of poultry. When lysine levels are too high or too low, this can lead to decreases in the rate of protein synthesis, increases in the rate of decomposition and decreased protein deposition, thus affecting the health and productivity of chickens (Bregendahl *et al*., 2008). The level of lysine in the feed not only reflects the quality of the feed but also serves as a reference for ideal amino acid models (Shao *et al*., 2018). This has a significant effect on enhancing daily weight gain and improving the feed conversion efficiency of animals. The demand for lysine is thus a key focus of research in the field of animal nutrition (Tian Dalong *et al*., 2018).

The hypothalamus plays a key role in regulating food intake and energy balance. Various neuropeptides produced by the hypothalamus promote or inhibit food intake. The regulation of feeding behavior in poultry is controlled by both the peripheral and central nervous systems. Nutritional levels and environmental factors affect metabolism and lead to the production of signal molecules such as neuropeptides, hormones and metabolites. These signal molecules bind to specific receptors and activate signal cascades of specific neuronal groups in the hypothalamus, thereby regulating feed intake and energy consumption in poultry at the cellular and systemic levels. Amino acids have been extensively studied in nutritional regulation, as they play central roles in protein synthesis and energy production. There is also significant interest in the physiological role of amino acids in the central nervous system, with the intake of feed in mammals and poultry being regulated by the hypothalamus (Tran *et al*., 2016).

Khan *et al*. (1979) suggested that the control of amino acids in the hypothalamus could be relevant to feeding regulation. Injecting a balanced mixture of amino acids into the hypothalamic soft tissue led to anorexia and reduced food intake (Khan *et al*., 1979), indicating that the amino acid levels in the brain may constitute a protein or amino acid availability signal detected by the nutrient-sensing region of the brain, forming the hypothalamic brainstem pathway. However, there is a lack of research on the molecular pathways by which lysine regulates feed intake in chicks through the hypothalamus. The mechanism underlying this phenomenon remains unclear.

To investigate the regulatory mechanism of lysine on feed intake in chicks, this study used Yao chickens as a research model. The effect of lysine on food intake was observed by adjusting the levels in the feed. Then, the hypothalamus tissues of the chicks were analyzed using RNA-seq and metabolomics to identify relevant genes. We applied Gene Ontology (GO) analysis and identification of signaling pathways to characterize the molecular regulatory pathways of lysine involved in poultry feeding.

MATERIALS AND METHODS

The study used chicks (Yao chickens) provided by the research chicken farm of Guizhou University. All animal experiments were conducted following the guidelines provided by the Animal Ethics Committee of Guizhou University.

The premix and amino acids were purchased from Jinmanchuan Feed Co., Ltd. (Guizhou, China). The restriction endonuclease was purchased from Shanghai Shenggong Biotechnology Co., Ltd. (Shanghai, China) and the primers were synthesized by the same company.

Four hundred healthy 1-day-old chicks (Yao chickens) were selected and randomly distributed into four groups, with five replicates per group (n = 20). Lysine levels were set as 0.65% (abbreviated as MGO), 0.85% (FIH), 1.0% (MGS) and 1.2% (FIL). The feeding experiment was conducted for a period of 6 weeks (1-42 days) and the research period was from June to July 2022. After weighing the day-old chicks, they were divided into groups and fed pre-weighed feed. The chicks were provided with feed and water ad libitum and the remaining amount of feed was recorded every day. At weeks 2, 4 and 6, the chicks were weighed and the data were used to calculate the average daily weight gain, daily feed intake, cumulative weight gain and cumulative feed intake during the experimental period. The data were analyzed using SPSS 18.0 statistical analysis software (IBM SPSS Ltd./Chicago USA) for one-way ANOVA and Duncan's test. Multiple comparisons were performed and the results are represented as mean ± standard deviation. $P<0.05$ indicates significant differences, while $P<0.01$ indicates extremely significant differences.

Six-week-old chickens were chosen from the highest and lowest feed intake groups and sacrificed. The birds were dissected on ice and the hypothalamus was removed, rinsed with DEPC water and placed in an enzyme-free centrifuge tube containing RNA preservation solution. The tubes were stored in liquid nitrogen and then transferred to an ultra-low temperature refrigerator at -80°C for storage and later use. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the RNA. The integrity of total RNA in the sample was detected using agarose gel electrophoresis and NanoDrop spectrophotometer was used to assess RNA purity (OD260/280), while an Agilent 2100 bioanalyzer was employed to determine RNA integrity. For the analysis, RNA samples with OD260/OD280 ratios between 1.8 and 2.1 and RNA integrity numbers greater than 8.0 were selected. OneStep kit was used for RNA sequencing.

Equal amounts of RNA from the same group were combined to create two mixed RNA samples. The samples were sent to Nuohe Biological Information Company (Beijing, China) for the construction of a cDNA library and sequencing. All samples underwent paired-end sequencing (2,150 bp) on an Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA).

Quality testing of raw sequencing data involved using the FastQC program to eliminate low-quality data with a quality score of less than 28, as outlined by Andrews (2010). Next, Trimmatic version 0.35 was used to filter the data by removing reads with adapters and low-quality reads (those with a base number of Qphred ≤20 accounting for more than 50% of the entire read length). The parameters were set to ILLUMINACLIP for adapters, fasta: 2:20:10, TRAILING: 20 and MAXINFO: 120:30 (Bolger *et al*. 2014) and the Q20, Q30 and GC content of the filtered data were calculated. The error rate of single amino group sequencing was less than 1%. The processed reads were compared with the chicken reference genome using HISAT2 version 2.0.4. The chicken reference genome (Galgal4) and annotation files were sourced from the NCBI database (https://www.ncbi.nlm.nih.gov/). Finally, genes with differential expression were identified using the quartile normalization method on FPKM to enhance the magnitude of differential expression.

Pathway annotation analysis was conducted on genes that exhibited differential expression to identify the biochemical metabolic pathways and signal transduction involved. Cluster profile software was used to conduct GO functional enrichment analysis on the set of genes that were differentially expressed using a threshold of padj <0.05 to identify significant enrichment. KEGG pathway enrichment analysis was performed on the set of differentially expressed genes using a significant enrichment threshold of padj <0.05.

A cDNA library was synthesized using a First Strand cDNA Synthesis Kit (Thermo Fisher, Waltham, MA, USA). Then, RT-PCR For the repeated RT-PCR analysis, each sample was subjected to six independent runs. Primer Premier 5.0 software was used to design real-time fluorescence quantitative PCR primers for the target gene and internal reference gene mRNA sequences published in previous studies. To validate the sequencing outcomes, we selected six DEGs for qRT PCR verification, using β-actin as an internal reference gene. The samples used were the same as for RNA-seq. The thalamic cDNA was used as a template for three repeated qRT-PCRs on the CFX96 system. The thalamic cDNA was used as the amplification template, with three replicates for each sample and three replicates as blank controls for the quantification of mRNA expression levels. β-Actin was used as an internal reference gene. Relative expression levels were quantified using the $2^{-\Delta\Delta}$Ct method.

The hypothalamic tissue (100 mg) was collected from chicks in the FIH (the highest food intake) and FIL (the lowest food intake) groups. This tissue was ground with liquid nitrogen. Then, the samples were transferred to enzyme-free EP tubes and added with 500 μL of an 80% methanol aqueous solution. After vortexing, the tubes were placed in an ice bath for 5 minutes and centrifuged at 15000 × g, 4°C for 20 minutes. A specified quantity of supernatant was diluted with mass spectrometry-grade water to achieve a methanol concentration of 53%. The solution was centrifuged at 15000 × g, 4°C for 20 minutes; the supernatant was collected and a SCIEX QTRAP ® 6500+mass spectrometry platform was employed for LC-MS detection. The raw gas mass spectrometry data were deconvolved using AMDIS software. The software Metabolyst was employed for pathway and metabolome analyses of differential metabolites, with P<0.05 indicating significant enrichment.

RESULTS AND DISCUSSION

Phenotypic measurement

The results of the phenotypic measurement are presented in Table 1. The FIH group exhibited a significantly higher average daily feed intake during the 0-2 week stage than the FIL group (P<0.05). At 2-4 weeks of age, the FIL group had a significantly lower average daily feed intake than the MGO, FIH and MGS groups (P<0.01). Additionally, there was no significant difference between the FIH group and the MGO and MGS groups (P>0.05). At 4-6 weeks, the average daily feed intake of the MGO and FIL groups was

Table 1: Effect of lysine levels on feed intake in Yao chickens during the 0-6 week period.

Lysine level	ADFI (0-2 W)	ADFI (2-4 W)	ADFI (4-6 W)	ADFI (0-6 W)
MGO	10.63±0.31^{ab}	21.56±1.08^{A}	36.91±2.25^{AB}	23.29±1.17^{A}
FIH	11.12±0.71^{a}	24.64±0.86^{A}	38.58±3.09^{A}	24.93±1.33^{A}
MGS	10.77±0.14^{ab}	23.99±0.86^{A}	39.94±1.68^{A}	24.78±0.73^{A}
FIL	10.31±0.46^{b}	17.32±1.61^{B}	33.26±2.78^{B}	20.13±1.41^{B}
P-value	0.134	≤0.001	0.005	≤0.001
Linear P-value	0.225	0.004	0.170	0.040
Secondary P-value	0.051	≤0.001	0.002	≤0.001

In the same column of data, the presence of different uppercase or lowercase letters indicates significant differences at P<0.01 and P<0.05, respectively. The absence of uppercase letters or the presence of identical uppercase letters indicates no significant differences (P>0.05).

significantly lower than that of the FIH and MGS groups (P<0.01). The difference between the FIH and MGS groups was not significant (P>0.05), nor was the difference between the MGO and FIL groups. Up to 6 weeks of age, the average daily feed intake of the FIL group was significantly lower than that of the MGO, FIH and MGS groups (P<0.01), with the average daily feed intake of the FIH group being the highest and not significantly different from that of the MGO and MGS groups (P>0.05).

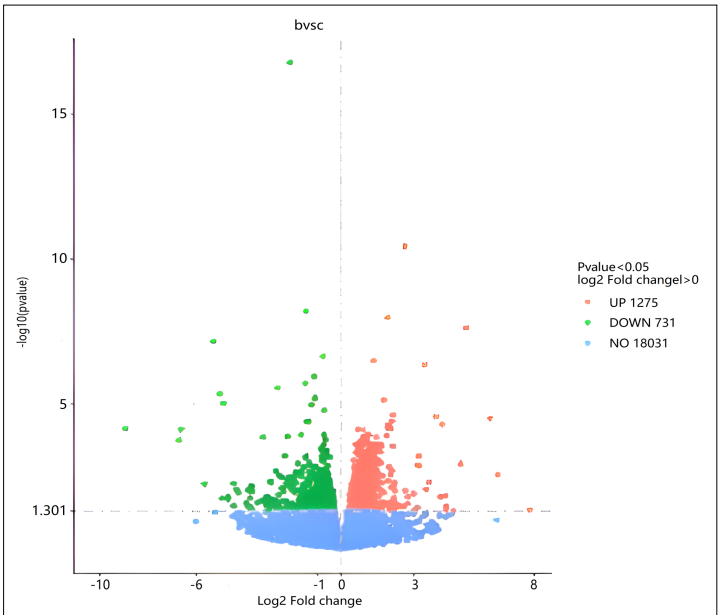
Differentially expressed genes

A total of 2006 differentially expressed genes were identified, com-prising 1275 upregulated genes and 731 downregulated genes (Fig 2), whose differential expression shown in Fig 1. For the validation of sequencing results, we selected six genes related to food in-take that were differentially expressed: SS 2, hypocretin (orexin) neuropeptide precursor (HCRT), AG-RP, CCKAR, NPY and CARTPT. The results were verified using qRT PCR and were consistent with the transcriptome sequencing results (Fig 2), thus indicating the accuracy of the sequencing results.

Functional analysis

Cluster profile software was used to perform functional enrichment analysis of 2006 genes that were differentially expressed, with the entire genome serving as the background. A total of 5794 terms were enriched, with 4423 terms being significantly enriched. There were 41 terms enriched in biological process (BP). Cellular component (CC) was significantly enriched in 26 terms, with a total of 536 terms. There were 835 terms enriched in molecular function (MF), with significant enrichment in four terms. The 10 most significant terms in BP were regulation of neurogenesis, projection of neuronal morphogenesis (GO: 0048812), active regulation of synaptic transmission (GO: 0050806), cell morphogenetic projection of the plasma membrane (GO: 0120039), cell morphogenetic projection (GO: 0048858), negative regulation of cell development (GO: 010721), glutamate receptor signaling pathway (GO: 007215), negative regulation of neurogenesis (GO: 0050768), cell morphoge-nesis part (GO: 0032990) and negatively regulated nervous system (GO: 0051961. The 10 most signi-ficant terms in CC were postsynaptic specialization (GO: 0099572), Receptor complex (GO: 0043235), inner mitochondrial membrane protein complex (GO: 0098800), postsynaptic density (GO: 0014069), asymmetric synapse (GO: 0032279), synapses between neurons (GO: 0098984), synaptic part (GO: 0044456), respiratory chain complex (GO: 0098803), plasma membrane receptor complex (GO: 0098802) and respiratory chain (GO: 070469). The four significant terms in MF were GTP enzyme binding (GO: 0051020), small GTP enzyme binding (GO: 0031267), Ras GTP enzyme binding (GO: 0017016) and guanine nucleotide exchange activity factor (GO: 005085). Ten bar charts for BP, CC, and MF are illustrated in Fig 3 and the KEGG enrichment characteristics were shown in Fig 4.

The biological processes related to chick feeding encompassed behavior (GO: 0007610), feeding behavior (GO: 0007631), cellular response to hormone stimuli (GO: 0032870), hormone-mediated signaling pathway (GO: 0009755), neuropeptide signaling pathway (GO: 0007218) and regulation of signaling receptor activity (GO: 0010469).



The horizontal axis in the figure represents the value of log2 FoldChange, while the vertical axis represents the value of -log10 P-value. The blue dashed line represents the threshold line for the criteria of differential gene screening.

Fig 1: Volcano map of differentially expressed genes.

The genes that were differentially expressed and closely related to feeding behavior, as mentioned earlier in the GO results, included growth hormone (GH), GRM1, GRM3, recombinant glutamate receptor, metabotropic 7 (GRM7), chromogranin A (CGA), HCRT, CCKAR, AGRP and NPY.

Using the entire genome as a background, cluster profiling was employed to perform KEGG pathway enrichment analysis of 2006 genes that showed differential

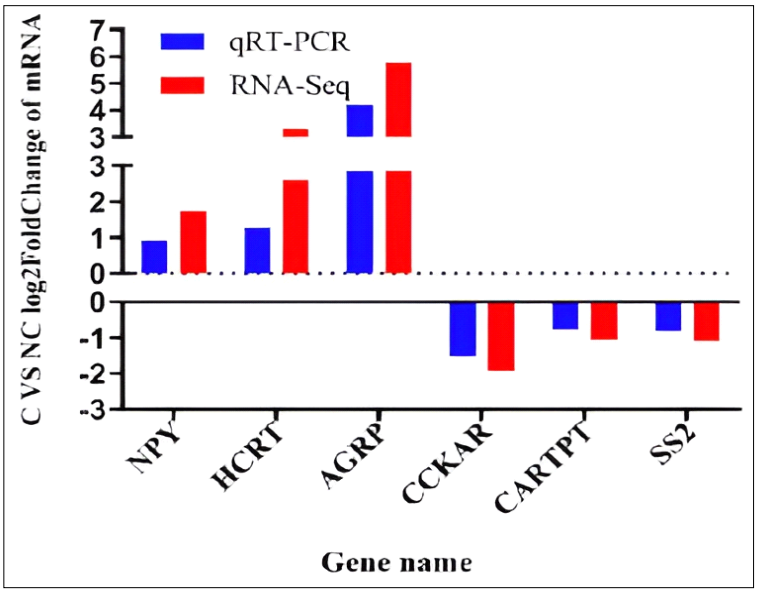
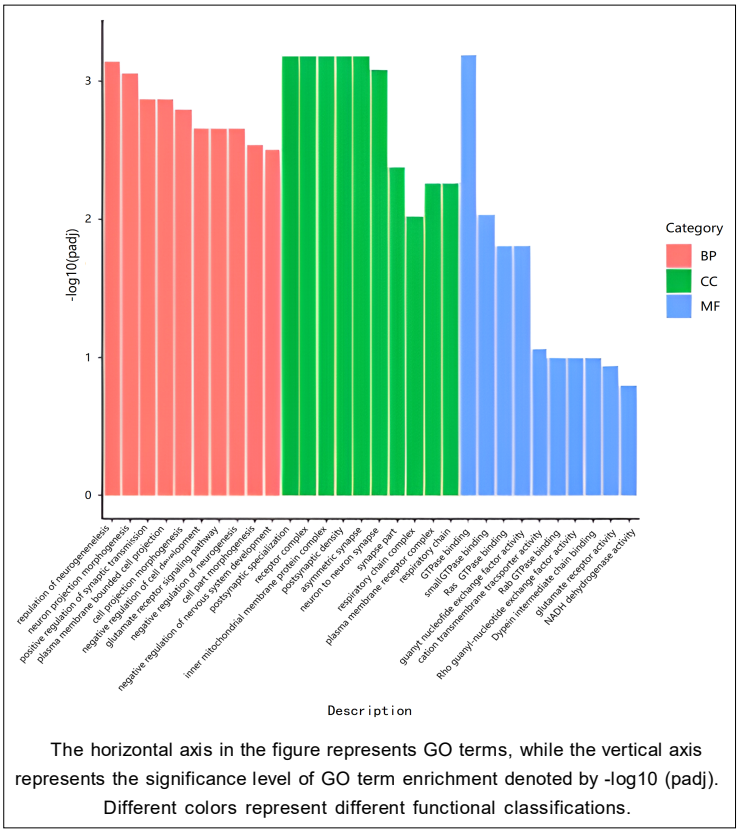


Fig 2: qRT-PCR was employed to verify the correlation between mRNA expression levels of differentially expressed genes and RNA-seq results.



The horizontal axis in the figure represents GO terms, while the vertical axis represents the significance level of GO term enrichment denoted by -log10 (padj). Different colors represent different functional classifications.

Fig 3: GO enrichment analysis histogram.

expression. The DEGs were enriched in 139 pathways, significantly enriched in seven pathways and highly enriched in 12 pathways. The analysis identified the following pathways: phosphatidylinositol signaling system (iga04070), ErbB signaling pathway (iga04012), focal junction (iga04510), regulation of actin cytoskeleton (iga04810), FoxO signaling pathway (iga04068), mTOR signaling pathway (iga04150), diabetes complications AGE-RAGE signaling pathway (iga04933), MAPK signaling pathway (iga04010), glutathione metabolism (iga00480), drug metabolism cytochrome P450 (iga00982), progesterone mediated oocyte maturation (iga04914), glyceride metabolism (iga00561), vascular smooth muscle contraction (iga04270), Apelin signaling pathway (iga04371), glycerophospholipid metabolism (iga00564), tight junction (iga04530), sheath phosphorus metabolism (iga00600) and gap junction (iga04540).

Differential metabolite screening

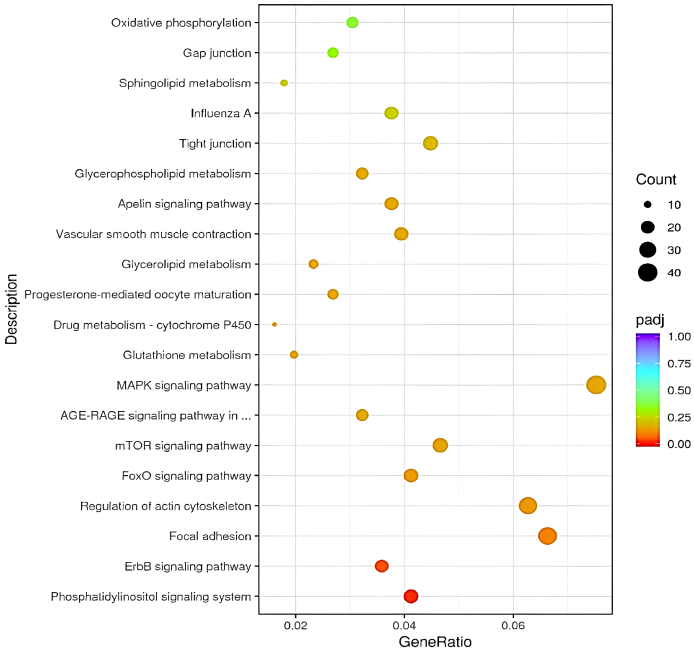
A total of 25 differential metabolites were screened by combining the VIP values (VIP >1) obtained from the PLS-DA model and the P-values obtained from a two-tailed Student's t-test (VIP >1.0, multiple of difference FC >1.2 or FC <0.833 and P-value <0.05). Of these 25 differential metabolites, nine were upregulated and 16 were downregulated, comprising nine types of metabolites. The differential metabolites are listed in Table 2.

Differential metabolite analysis

A hierarchical clustering analysis was performed on two groups of differential metabolites and the differences in metabolic expression patterns were determined between and within the two groups for each comparison. The analysis suggested that the differences in relative quantitative values of metabolites between the two groups were highly significant (Fig 5). Metabolite products present in the hypothalamic tissue of Yao chickens, including goose carnosine and branched-chain amino acids, may stimulate feed intake, leading to a higher intake in the FIH level group compared to MGO, MGS and FIL. Metabolic products such as 5-hydroxytryptophan may have hindered feed intake in the FIL group. The KEGG analysis identified differential metabolites enriched in 26 pathways, with ID map02010 and name ABC transporters significantly enriched in one pathway. The enriched metabolites included D-mannose, 2-aminoethylphosphonate and L-valine. The entire pathway enriched by differential metabolites is presented in Table 3.

Regulatory genes

RNA sequencing of the hypothalamus of chicks in the FIH and FIL groups was conducted. A bioinformatic analysis was conducted using the DESeq method, resulting in the screening of 2006 genes that exhibited differential



The horizontal axis in the figure represents the ratio of genes annotated onto the KEGG pathway to the total number of genes that exhibit differential expression. The vertical axis represents the KEGG pathway and the size of the dots represents the number of genes annotated. The colors ranging from red to purple signify the importance of enrichment.

Fig 4: KEGG enrichment scatter plot.

expression. Compared with the FIL group, the FIH group expressed 1275 genes whose levels were upregulated, along with 731 genes that were downregulated. The sequencing results were consistent with the RNA-seq results as verified by qRT PCR. The results suggested that lysine may play a crucial role in the feeding regulation of Yao chickens by adjusting the expression levels of differentially expressed genes. These genes related to food

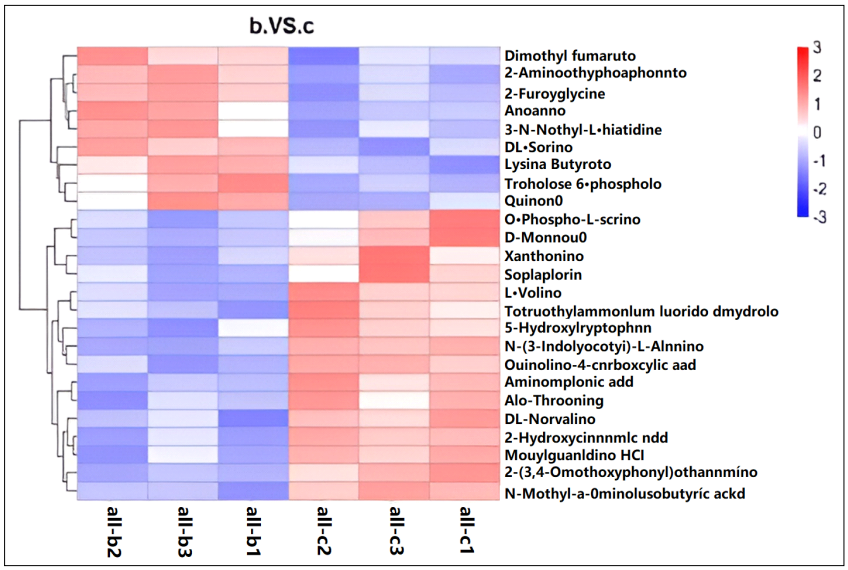


Fig 5: Cluster heat map of subgroups of differential metabolites.

Table 2: Differential metabolites between group FIH and group FIL.

Metabolite name	Molecular formula	Retention time (min)	P-value	Chemical classification	Up-regulated/ Down-regulated
Aminomalonic acid	$C_3H_5NO_4$	0.86	0.005	Organic acids and their derivatives	Down
2-(3,4-Dimethoxyphenyl) ethylamine	$C_{10}H_{15}NO_2$	1.77	0.005	Benzene and its derivatives	Down
N-(3-Indoleacetyl)-L-alanine	$C_{13}H_{14}N_{203}$	3.88	0.006	Amino acids and their derivatives	Down
L-valine	$C_5H_{11}NO_2$	1.2	0.007	Amino acids and their derivatives	Down
N-methyl-α-aminoisobutyric acid	$C_5H_{11}NO_2$	1.02	0.008	Organic acids and their derivatives	Down
Xanthine nucleoside	$C_{10}H_{12}N_{406}$	3.69	0.02	Nucleotides and their derivatives	Down
2-Hydroxycinnamic acid	$C_9H_8O_3$	1.71	0.022	Cinnamic acid and its derivatives	Down
L-allothreonine	$C_4H_9NO_3$	0.82	0.026	Amino acids and their derivatives	Down
Cytosine nucleoside	$C_6H_{20}FN$	1.21	0.027	Nucleotides and their derivatives	Down
Quinoline-4-carboxylic acid	$C_{10}H_7NO_2$	2.46	0.028	Organic acids and their derivatives	Down
L-O-phosphoserine	$C_3H_8NO_6P$	0.79	0.028	Amino acids and their derivatives	Down
D-n-valine	$C_5H_{11}NO_2$	1.06	0.029	Amino acids and their derivatives	Down
D-mannose	C_6H_{1206}	1.41	0.030	Carbohydrates and their derivatives	Down
5-Hydroxytryptophan	$C_{11}H_{12}N_2O_3$	3.17	0.042	Amino acids and their derivatives	Down
Sepiapterin	$C_9H_{11}N_5O_3$	0.84	0.044	Organic heterocyclic compounds	Down
1-Methylguanidine hydrochloride	$C_2H_8C_1N_3$	0.86	0.045	Organic acids and their derivatives	Down
2-Aminoethyl phosphate	$C_2H_8NO_3P$	0.88	0.007	Organic acids and their derivatives	Up
DL serine	$C_3H_7NO_3$	0.80	0.017	Amino acids and their derivatives	Up
N-(2-furoyl) glycine	$C_7H_7NO_4$	0.68	0.021	Amino acids and their derivatives	Up
Goose carnosine	$C_{10}H_{16}N_{403}$	0.81	0.023	Organic acids and their derivatives	Up
Trehalose-6-phosphate	$C_{12}H_{23}O_{14}P$	0.92	0.024	Carbohydrates and their derivatives	Up
L-lysine butyric acid	$C_{10}H_{22}N_{204}$	0.91	0.029	Amino acids and their derivatives	Up
3-N-methyl-L-histidine	$C_7H_{13}N_3O_3$	0.56	0.033	Amino acids and their derivatives	Up
Benzoquinone	C_6H_{402}	7.04	0.044	Ketones	Up
Dimethyl fumarate	$C_6H_8O_4$	0.91	0.045	Fatty acyls	Up

intake include NPY, HCRT, CGA, RLN3, PMCH, CCKAR, CCKBR, SS2, GH and NPBWR1.

The intake of food is directly linked to the growth and productivity of animals (Cantalapiedra Hijar *et al*., 2018; Singh *et al*., 2020). Food intake in animals is primarily controlled by the peripheral nervous system, the central nervous system and the endocrine system (Chen Lushuang *et al*., 2020). The hypothalamus regulates food intake through action on the central nervous system (Niknafs *et al*., 2018). The neuropeptides synthesized and secreted by the hypothalamus that regulate appetite play a crucial role in controlling the feeding behavior of animals. These neuropeptides include AgRP and NPY in pufferfish and mice and cocaine- and amphetamine-regulated transcriptional peptides (CARTPT). Changes in the nutritional composition of the diet may affect the secretion of these appetite-regulating neuropeptides, ultimately influencing the animal's food intake (Tian *et al*., 2019).

Schwartz *et al*. (2016) showed that appetite-promoting neuropeptides (NPY/AGRP) are present in the hypothalamus of poultry and that they play a role in regulating food intake by releasing signaling molecules. According to Sheng Wei *et al*. (2020), NPY is a crucial factor in the regulation of food intake. The qRT PCR validation in this study indicated that NPY expression in the hypothalamus was higher in the FIH group than in the FIL group and the feed intake of the FIH group was significantly greater than that of the FIL group. Hence, NPY serves as a promoting factor. He *et al*. (2019) examined the effect of heat stress on hypothalamic integrity, serum indicators and hypothalamic appetite gene expression in broilers. Their findings revealed that heat stress decreased the expression of the appetite gene NPY in broilers, subsequently leading to a reduction in feed intake, consistent with the results of this study. Bahry *et al*. (2017) examined the regulatory effect of central neuropeptide Y on monoamines and corticosteroids in chickens subjected to high temperatures and fasting. The results indicated that hypothalamic injection of NPY could counteract the reduction in food intake during heat stress and stimulate feeding in chickens, in agreement with the results of this study. AGRP is a neuropeptide that promotes food intake and it can directly sense changes in nutrients (sugars, fats and amino acids) (Xiang Nana, 2016), projecting these signals through neuronal axons to the hypothalamus and thereby regulating animal food intake (Bonilla *et al*., 2006). AGRP is a natural antagonist and agonist of melanocortical hormones. α-MSH competes with AGRP for binding to MC4R, inhibiting anorexia and promoting feeding (Chai *et al*., 2003). In this study, the expression level of AGRP was higher in the FIH group than

Table 3: Enrichment pathways of the differential metabolites from the KEGG database.

Map ID	Map title	P-value	X
map02010	ABC transporter	0.039	3
map00970	Aminoacyl-tRNA biosynthesis	0.050	2
map00232	Caffeine metabolism	0.062	1
map00440	Phosphonate and phosphinate metabolism	0.084	1
map04142	Lysosome	0.084	1
map04723	Retrograde endocannabinoid signaling	0.084	1
map00280	Valine, leucine and isoleucine degradation	0.122	1
map00290	Valine, leucine and isoleucine biosynthesis	0.122	1
map00790	Folate biosynthesis	0.122	1
map00770	Pantothenate and CoA biosynthesis	0.179	1
map00500	Starch and sucrose metabolism	0.205	1
map01230	Biosynthesis of amino acids	0.219	2
map00051	Fructose and mannose metabolism	0.233	I
map04978	Mineral absorption	0.232	1
map00410	β-Alanine metabolism	0.283	1
map05230	Central carbon metabolism in cancer	0.306	1
map00270	Cysteine and methionine metabolism	0.333	1
map00340	Histidine metabolism	0.333	1
map00520	Amino sugar and nucleotide sugar metabolism	0.333	1
map01210	2-Oxocarboxylic acid metabolism	0.333	1
map04974	Protein digestion and absorption	0.333	1
map00260	Glycine, serine and threonine metabolism	0.375	1
map00052	Galactose metabolism	0.408	1
map01200	Carbon metabolism	0.466	1
map01100	Metabolic pathways	0.671	7
map00230	Purine metabolism	1	1

in the FIL group. The FIL group's lower expression level of AGRP resulted in reduced food intake. SS 2, also referred to as SST 7, is a member of the somatostatin gene (SST) family that is predominantly expressed in avian autonomic neurons (Nishi *et al*., 2010). According to Stengel *et al*. (2015), somatostatin is a peptide hormone that consists of 14 amino acids and is primarily found in the nervous and digestive systems of animals. SST has a regulatory effect on the secretion of GH. During the growth process of animals, GH and insulin-like growth factors serve as the primary substances that regulate animal growth and development. Their secretion is doubly regulated by SST and GH-releasing factor (GRF). GRF can stimulate the release of GH, whereas SST can inhibit the release of GH (Prévost *et al*., 1996; Lin *et al*., 2001). Somatostatin, a well-known inhibitor of GH release (Brazeau *et al*., 1973), is widely distributed throughout the brain. Nakahara (2012) showed that feeding mice with a diet deficient in Val led to a notable elevation in hypothalamic somatostatin mRNA levels, suggesting that somatostatin may play a role in the regulation of amino acids. Nakahara *et al*. (2012) demonstrated that the injection of somatostatin into the brain significantly decreased food intake in mice.

In this study, the FIL group had elevated levels of lysine and demonstrated an amino acid imbalance in their diet. Furthermore, the expression level of SS2 was greater in the FIL group than in the FIH group, resulting in a reduction in feed intake of chicks. In this study, SS 2 was identified as a feeding suppressor gene. Cocaine- and amphetamine-regulated transcript, also referred to as CART, is a pituitary peptide hormone that is under the strict control of GnRH in the hypothalamus for its expression and secretion. The hormone belongs to the category of anorexic neuropeptides and is an active molecule in the hypothalamic-pituitary-gonadal axis, with potential endocrine regulatory functions. CART is composed of either 41 or 48 amino acids and is present not only in hypothalamic tissue but also in the intestine. It plays a crucial role in animal feeding and the regulation of energy balance. Intracerebral injection of CART inhibits both gastric acid secretion and gastric emptying (Mo *et al*., 2019). In their 2012 study, discovered that the injection of CART could suppress food intake in mice, leading to a significant increase in weight gain in the group without CART injection compared with the injection group. Research has shown that CART may directly affect body weight by mediating the activation of serotonin 4 receptors and influencing food intake. CART regulates the expression of thyrotropin-releasing hormone in the hypothalamus and the release of thyrotropin in the pituitary gland, which increases the consumption of brown adipose tissue, thereby influencing energy homeostasis and ultimately food intake (Jean *et al*., 2007). This study employed qRT PCR to confirm that the expression of CARTPT in the hypothalamus was lower in the FIH group than in the FIL group, while the feed intake was higher in the FIH group than in the FIL group. CARTPT is thus an inhibitory factor in feed intake.

This study employed qRT PCR to confirm that the expression of CCKAR in the hypothalamus was lower in the FIH group than in the FIL group, while the feed intake was higher in the FIH group than in the FIL group. CCKAR inhibits feed intake. The sequencing results showed that the expression level of CCKBR in the hypothalamus of the FIH group was lower than that of the FIL group, while the feed intake of the FIH group was higher than that of the FIL group (Schwartz, 2000; Smith, 2012; Tominaga *et al*., 2020). Specifically, CCK activates visceral sensory afferent receptor signals that send satiety signals to the hypothalamus, causing feeding to stop. According to Baptista *et al*. (2005), CCK regulates food intake by inducing vagal activity mediated by the activity of glucagon-like peptide neurons in the hypothalamus. The effect of CCK on food intake may involve melanocortin receptors and melanocortin signaling in the dorsal vagus nerve of the hypothalamus, as suggested by van den Pol (2003). Hence, CCK assumes a pivotal role in the regulation of feeding behavior through its involvement in visceral sensory inputs and potential signaling mechanisms that are also used by glucagon-like peptides, leptin and other factors associated with the regulation of feeding behavior (Williams *et al*., 2012). Employed transcriptome technology to sequence the hypothalamus of broiler chickens fed with steviol. They discovered that steviol could stimulate feed intake by modulating the ligand-receptor interaction pathway of hypothalamic nerve activity. Conversely, NPY, NPY5R and TSHB are known to stimulate feed intake, whereas NMU, TPH 2 and DDC inhibit feeding. NPY, a neuropeptide, plays a crucial role in regulating feed intake in broilers by interacting with several receptor subtypes, including NPY, NPY2R and NPY5R. TSHB plays a vital role in the synthesis and secretion of thyroid hormones by activating the cAMP signaling pathway. NMU may induce anorexia in chickens by upregulating corticotropin-releasing hormone and arginine angiotensin in the central nervous system. The downregulation of TPH2 and DDC expression may impede dopamine production, resulting in an increase in food intake. The inclusion of steviol may regulate appetite signals in the hypothalamus, thus promoting feed intake (Jiang *et al*., 2021). Conducted transcriptome sequencing of the hypothalamus of chickens that were injected with exogenous adiponectin. They discovered that feeding-related differential genes were significantly enriched in the neuroactive ligand-receptor interaction pathway. Furthermore, adiponectin may stimulate appetite and increase food intake *via* the POMC/CRH and NPY/AGRP signaling pathways. The expression of NPY and AGRP increases with the increase in adiponectin dosage and adiponectin may directly or indirectly upregulate NPY and AGRP to promote feed intake in chicks. The result may also be due to the downregulation of POMC to inhibit dopamine secretion and the downregulation of CRH to affect the HPA axis to promote appetite. This study has preliminarily confirmed the molecular mechanism through which

adiponectin regulates food intake *via* the hypothalamus (Li *et al*., 2018). The interaction pathway of ligand-receptors in hypothalamic nerve activity plays a crucial role in regulating the feed intake of Yao chickens. At 0–6 weeks of age, high concentrations of lysine may directly or indirectly downregulate genes such as NPY and AGRP, upregulate the expression of the CARTPT gene and affect the HPA axis to curb appetite and regulate the feed intake of chickens.

Marked metabolites

Using LC-MS technology, this study examined the hypothalamic tissue of Yao chickens and investigated alterations in hypothalamic metabolites after 6 weeks of feeding varying levels of lysine. Through PLS-DA analysis, a total of 25 differential metabolites were obtained from groups FIH and FIL, including organic acids and their derivatives, amino acids and their derivatives and nucleotides and their derivatives. These metabolites were enriched in 26 pathways and significantly enriched in the ABC transporters pathway. According to a study conducted by de Haas *et al*. (2018), serotonin is closely associated with the feeding behavior of chickens. This study indicates that lysine can regulate the appetite of broilers via serotonin and that a high content of lysine can decrease their feed intake. According to Hui *et al*. (2020), 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter that regulates the function of both the central and peripheral nervous systems. Central serotonin (5-HT) is a neurotransmitter responsible for anorexia. After being released, serotonin exerts its effects by binding to serotonin receptors (HTRs), most of which are G protein-coupled receptors. According to De *et al*. (2018), serotonin plays a significant role in the regulation of the hypothalamus in chickens through various pathways, including blood, hormones and the vagus nerve. In poultry, serotonin inhibits appetite, leading chickens to feel satiated (Gillette, 2006). The regulation of decreased appetite by serotonin is primarily attributed to the hypothalamus. Specifically, serotonin enhances satiety and reduces food intake by influencing the hindbrain and hypothalamic circuits that regulate the stimulation of peripheral signals indicating energy status (Pratt *et al*., 2021). The higher expression of 5-hydroxytryptophan in the hypothalamus of Group C than in Group B may be due to the activation of opioid precortical hormone neurons through the 5-HT2C receptor and the inhibition of NRY/AGRP-related protein in neurons through the 5-HT1B receptor, resulting in reduced food intake (Heisler *et al*., 2002). Zendehdel *et al*. (2017) injected serotonin into the hypothalamus of chicks to reduce their feed intake. This was achieved through the regulation of adrenaline α 2 and β 2 receptors responsible for controlling the feeding behavior. The regulation of food intake, which primarily occurs in the hypothalamus, is a crucial process influenced by alterations in nutrient levels within the animal brain, as noted by Delgado *et al*. (2017). The hypothalamus senses related hormone signals and is equipped with multiple nutrient perception mechanisms that can detect changes in the levels of glucose, fatty acids and amino acids (Soungas *et al*., 2018). Valine plays a crucial role in the hypothalamic regulation of food intake. It is not metabolized directly into acetyl CoA but rather into propionyl CoA that is then converted into succinyl CoA and enters the tricarboxylic acid cycle. This glucose-type amino acid regulates glucose metabolism by acting on the hypothalamus, thereby enhancing satiety and reducing food intake (Arrieta Cruz *et al*., 2016). In this study, the feed intake of Group C was lower than that of Group B and the expression of the metabolite valine was higher in Group C than in Group B. Valine may serve as a marker metabolite for high lysine levels, an amino acid known to inhibit feed intake in Yao chickens.

CONCLUSION

A total of 2006 differentially expressed genes were obtained from the highest and lowest intake groups, comprising 1275 upregulated genes and 731 downregulated genes. The KEGG and GO functional enrichment analyses revealed that the genes with differential expression were significantly enriched in 139 signaling pathways and 5794 terms. The expression of genes such as SS2, CCKAR, CCKBR and NPY/AGRP in the hypothalamus is regulated by excessive lysine that acts on the neural active ligand-receptor interaction pathway and influences the feed intake of Yao chickens during the breeding period.

Recent studies have discovered that 5-hydroxytryptophan and L-valine serve as lysine biomarkers that influence the hypothalamic regulation of feed intake in Yao chickens. When the concentration of lysine is too high, the hypothalamus increases the levels of aminomalonic acid, 2-(3,4-dimethoxyphenyl)-ethylamine, N-(3-indoleacetyl)-L-alanine, L-valine and N-methyl-α-aminobutyric acid. Additionally, the expression levels of amino isobutyric acid, xanthine nucleoside, 2-hydroxycinnamic acid, L-allothreonine, cytosine nucleoside, quinoline-4-carboxylic acid, L-O-phosphoserine, D-n-valine, D-mannose, 5-hydroxytryptophan, methotrexate and 1-methyl guanidine hydrochloride metabolites are increased. By inhibiting the expression of metabolites such as 2-aminoethyl phosphate, DL serine, N(2-furoyl) glycine, goose carnosine, trehalose-6-phosphate, lysine butyrate, 3-N-methyl-L-histidine, benzoquinone and dimethyl fumarate, it is possible to regulate the expression of related genes through the ABC transporters metabolic pathway. This in turn can affect feed intake during the 0-6 week period and subsequently influence the growth, development and production performance of chickens.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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