Supplementing Genistein for Breeder Hens Alters the Fatty Acid Metabolism and Growth Performance of Offsprings by Epigenetic Modification

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The experiment was designed to clarify the effect and molecular mechanism of maternal genistein (GEN) on the lipid metabolism and developmental growth of offspring chicks. Laying broiler breeder (LBB) hens were supplemented with 40 mg/kg genistein (GEN), while the control group was fed with the low-soybean meal diet. The offspring chicks were grouped according to the mother generation with 8 replicates each. Hepatic transcriptome data revealed 3915 differentially expressed genes (DEGs, \textit{P} adjusted < 0.05, fold change > 1.5 or fold change < 0.67) between chicks in the two groups. Maternal GEN activated the GH-IGF1-P13K/Akt signaling pathway, which promoted the developmental processes and cellular amino acid metabolic processes, as well as inhibited the apoptotic process. GEN treatment significantly increased the weight gain, breast muscle percentage, and liver index in chicks. PANTHER clustering analysis suggested that maternal GEN enhanced the antioxidant activity of chicks by the upregulation of gene (SOD3, MT1, and MT4) expression. Accordingly, the activities of T-AOC and T-SOD in the liver were increased after GEN treatment. The overrepresentation tests revealed that maternal GEN influenced the glycolysis, unsaturated fatty acid biosynthesis, acyl-coenzyme A metabolism, lipid transport, and cholesterol metabolism in the chick livers. Hepatic cholesterol and long-chain fatty acid were significantly decreased after GEN treatment. However, the level of arachidonic acid was higher in the livers of the GEN-treated group compared with the CON group. Moreover, GEN treatment enhanced fatty acid \(\beta\)-oxidation and upregulated PPAR\(\delta\) expression in the chick liver. ChIP-qPCR analysis indicated that maternal GEN might induce histone H3-K36 trimethylation in the promoter region of PPAR\(\delta\) gene (PPARD) through Iws1, methyltransferases. It also induced histone H4-K12 acetylation at the PPARD promoter through MYST2, which activated the PPAR signaling pathways in the chick livers. In summary, supplementing LBB hens with GEN can alter lipid metabolism in the offspring chicks through epigenetic modification and improve the antioxidative capability as well as growth performance.

1. Introduction

Maternal nutrition during gestation has potentially adaptive effects on the phenotype of animal offspring [1]. Programmed metabolic adaptations that occur in utero alter postnatal growth and body composition [2, 3]. Studies identified that placental changes during gestation can induce histone H3 hyperacetylation in the liver of rat offsprings [4]. Epigenetic adaptations occurring during the early developmental period are associated with increased susceptibility for adult-onset metabolic diseases [5, 6]. Thus, epigenetic modifications induced by nutritional changes in the embryo and neonate may influence metabolic status during adulthood. Histone acetylation regulates many cellular processes, but single or multiple acetylation sites cause different effects [7]. Acetylation has been suggested to activate gene transcription, whereas deacetylation mainly represses transcription. Histone methylation is a dynamic change that is strictly regulated by histone methylase and demethylase [8]. This change plays a critical role in transcriptional regulation, genome stability, disease development, developmental growth, and chromatin remodeling, which is gradually erased.
during the early embryonic period [9, 10]. However, recent studies revealed that histone methylation also be
cross-generationally inherited [11, 12]. Therefore, maternal nutrition that affects epigenetic patterning can potentially influence the adult phenotype via alterations in transcriptional promoters.

Genistein (GEN, 4′,5,7-trihydroxyisoflavone), a type of
isoflavone (ISF), exists widely in soybean products. It has
been used for the treatment of cancer, cardiovascular, and
estrogen-dependent diseases [13]. GEN also reportedly ac-
vated peroxisome proliferator-activated receptors (PPARs)
[14]. PPARs can combine with the retinoid X receptor to
form a heterodimer, which combines with PPAR response
element in the promoter region [15]. Studies suggest that
PPARs can promote fatty acid catabolism by enhancing
fatty acid transport and β-oxidation [16]. Accordingly, ISFs
reportedly decrease plasma triglyceride (TG) and choles-
terol (CHOL) levels in hens fed with high-fat diets along
with an increased expression of PPARs [17]. The phenolic
hydroxyl in the structure of GEN can bind oxygen-free radicals as hydrogen donors. The antioxidant activity of GEN is stronger than ascorbic acid and quercetin [18]. It is
reported that 40 or 80 mg/kg dietary ISF increases the antio-
oxidant capability (T-AOC) and superoxide dismutase (T-
SOD) activity of chick plasma [19]. As a DNA methyltrans-
ferase inhibitor, ISFs can reduce DNA (cytosine-5)-methyl-
transferase 3B expression. Recent research indicated that
GEN treatment on maternal mice alleviated obesity along
with DNA epigenetic modification, and this effect can con-
tinue until offspring adulthood [20]. ISFs also reportedly inhibit cervical cancer, prostate cancer, and oesophageal
cancer by regulating histone acetylation and DNA methyl-
ation [21–23].

Previous studies in our laboratory have indi-
cated that supplementing 40 mg/kg GEN for LBB hens
significantly increased hepatic PPARδ expression, which
regulates fatty acid metabolism in embryos. Interestingly,
the effects of maternal GEN on the metabolism, growth,
and antioxidiant status of hatched chicks are still unknown.
Additionally, its effects on histone modification in progeny
are currently rarely reported.

The nutritional supply for mammalian foetuses depends
on the maternal metabolism and placental transport [24].
However, the development of chick embryos and newly
hatched chicks entirely depends on the nutrients deposited
in the eggs. GEN in the body exists mainly in the form of
sulfate and glucuronide, and dietary ISF for laying hens
reportedly can be deposited in the egg yolk [25]. LBB hens
during the late egg-laying period were sensitive to estrogen-
like substances, and the phenotypic data of their offspring
are plentiful and repeatable. Therefore, we chose birds in
two generations as the animal model. In the current
research, we used RNA-Seq to study the effects of maternal
GEN on the hepatic transcriptome of offspring chicks, aim-
ing to explore the changes in lipid metabolism, growth per-
formance, and antioxidiant status. Furthermore, we used the
ChIP-qPCR technology to clarify the regulatory mechanism
of maternal GEN effect on the key metabolism-related
genes in relation to histone modification.

2. Materials and Methods

2.1. Materials. GEN is a synthetic product from Kai Meng
Co. (Xi An, China) Chemical Plant with 99.8% purity.

2.2. Animals and Experimental Design. All procedures for an-
imal handling were conducted under protocols approved by
the Animal Welfare Committee of China Agricultural Univer-
sity (CAU/NO.160515-2). The experiment was carried out on
LBB hens and male offspring chicks at a commercial farm
(Zhuozhou, China) under standard conditions. The adapta-
tion period lasts for 2 weeks. Then, 480 57-week-old Ross
308 LBB hens were allocated into two groups (Ab and Bb) with
8 replicates of 30 hens. As shown in Table S1, the corn-soybean
meal-cottonseed meal (CSCM) diet was formulated to meet
the nutritional requirements of LBB hens according to the
Nutrient Requirements of Poultry (NRC, 1994) and previous
research [26]. The LGE and HGE groups were fed with the
CSCM supplemented with GEN at 40 and 400 mg/kg, respec-
tively. The Ab and Bb groups were fed with CSCM diet supple-
menting with GEN at 0 and 40 mg/kg, respectively.
The formal experiment period lasts for 8 weeks. Each hen
was allotted 155 g of feed at 6:00 a.m. every day, with free
access to water. Male breeders were caged and given a
commercial diet. Eggs (20 eggs each replicate) from 65-
week-old LBB hens were incubated under 70–80% humidity
and 37.8 °C. According to the treatment on the maternal
generation, chicks were divided into 2 groups (CON and
GEN) with 8 replicates (cages) of 10 birds each. The chicks
with free access to water and diet were housed in a standard
house, which continued to 3 weeks. All chicks were fed with
the corn-soybean meal diet (Table S1), which was
formulated based on NRC (1994).

2.3. Sample Collection and Chemical Analysis. Body weight
gain (BWG), feed intake (FI), and feed conversion ratio
(FCR) were determined by all broilers in each replicate. On
the 21st day of the broiler-raising experiment, one chick from
each cage, with a body weight close to the average, was
selected for the wing vein blood collection and slaughter after
8 hours of feed deprivation. Serum was centrifuged at 3000 ×
g for 10 min and stored at -20 °C until further use for bio-
chemical analysis. Liver samples for RNA-Seq and metabolic
analysis were collected, which were frozen in liquid nitrogen
and kept in a freezer (-80 °C) immediately. In addition, one
another broiler from each replicate, closing to the average
bodyweight, was selected for measurements of carcass traits.

2.4. Measurements of Serum Biochemical Indexes. Serum TG,
TC, and HDL/LDL (high/low density lipoprotein) levels were
assayed using assay kits (Unicel DxC 800, Beckman Coulter,
California, America). Serum triiodothyronine (T3), tetraio-
dothyronine (T4), and growth hormone (GH) levels were
measured using commercial double-antibody radioimmuno-
assay kits purchased from Shanghai Institute of Biological
Products. The interassay coefficient of variation was 10%.

2.5. Measurement of Antioxidant and Fatty Acid Metabolic
Indexes. Liver samples were homogenized with saline to
make a 10% homogenate. The malondialdehyde (MDA)
levels; T-SOD, CAT, and glutathione peroxidase (GSH-Px) activities; and T-AOC of the 10% homogenate were determined by using a kit (Nanjing Jiancheng Inc., China) according to the manufacturer’s protocol. Lipids were extracted according to the method of Bligh and Dyer for further analysis of long-chain fatty acids (LCFAs) [27]. Liver TG and cholesterol levels were examined using commercially available colorimetric diagnostic kits (Nanjing Jiancheng Bioengineering Institute, China). The detection of LCFAs in the liver was conducted referring to the method in our previous research [28].

2.6. RNA-Seq Analysis

2.6.1. RNA Isolation, Library Preparation, and Sequencing. Total RNA was extracted and purified from frozen liver tissues using TRIzol (transgene) according to the manufacturer’s protocols. Total RNA was then quantified using a nucleic acid/protein quantitative measuring instrument (Bio-Rad). Then, the samples were pooled into one per group at a standardized concentration. Next, four RNA samples from each of the four replicates per treatment were packed in dry ice and sent to Macrogen Millennium Genomics for further library preparation and sequencing. RNA-Seq was performed on an Illumina HiSeq 2500 platform using pair-end sequencing with a read length of 126 bp.

2.6.2. Sequence Read Quality Control. According to the method of quality control in the previous reports, raw reads were assessed using FastQC (version 0.10.1) [29, 30]. Reads with adapter, fuzzy N bases, rRNA, sequences shorter than 20 nt, and low quality with $Q<20$ were trimmed with fastX clipper (version 0.0.13) [31]. All 126 bp double-end reads of 8 samples from 2 groups were separately aligned to the chicken reference genome (Gallus gallus 4.0, version 81, Ensembl), using the spliced mapping algorithm in TopHat2 (version: 2.0.9) [32]. Subsequently, reads were counted by gene using HTSeq and Gallus gallus genome GTF file (Gallus gallus 4.0, version 81, Ensembl). All software runs with the default parameters.

2.6.3. Identification of Differentially Expressed Genes and Gene Ontology Terms. Gene expression intensity was calculated using the reads per kilobase per million (RPKM). Differentially expressed genes (DEGs) were analyzed using Cufflinks software [33]. The DEG model library was fitted to library type and treatment effect. DEGs and $P$ values were determined using tests based on negative binomial distribution. All obtained $P$ values were adjusted for the false discovery rate ($q$-value) with multiple testing procedures used to control for type I errors [34]. Genes with expression levels with a $P$ adjusted < 0.05, fold change > 1.5 or fold change < 0.67 between two samples were defined as DEGs. We functionally classified identified DEGs using the PANTHER classification system (http://www.pantherdb.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp/kegg/) database.

2.6.4. RNA-Seq Confirmation and Measurement of DNA Enriched by qRT-PCR. To confirm the sequencing data, qRT-PCR was performed on 17 randomly selected DEGs using similar methods in the previous report [28]. Total RNA was isolated from tissue samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Total RNA was reverse-transcribed to cDNA using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa) according to the manufacturer’s instructions. The one-step real-time RT-PCR was performed using SYBR Premix Ex Taq™ (TaKaRa, Dalian, LiaoNing, China) in a real-time PCR machine (ABI7500; Applied Biosystems, Carlsbad, CA, USA) following the manufacturer’s guidelines. Primers were designed via Primer Express 3.0.1 software (Applied Biosystems) and are shown in Table S2. GAPDH was used as the housekeeping gene. Relative mRNA expression levels of each target gene were normalized to the control using the $2^{-\Delta\Delta Ct}$ method. The DNA enriched by ChIP was amplified by qPCR. The information of primer at the two sites of the promoter region of gene PPARD was shown in Table S3.

2.7. Assay Procedure of Chromatin Immunoprecipitation (ChIP). We conducted the assay according to the method of Saleh et al. [35]. The whole procedure included chromatin crosslinking with tissue samples, chromatin fragmentation, chromatin immunoprecipitation, reverse crosslinking, and DNA purification. The antibodies used in the assay include anti-acetyl-histone H4 (Lys12) (07-595, Millipore, US), anti-acetyl-histone H4 (Lys 8) (A7258, AbClnal, US), anti-acetyl-histone H3 (Lys 4) trimethylation (04-745, Millipore, US), and anti-acetyl-histone H3 (Lys 36) (ab9050, AbClnal, US) antibodies. The improved method is shown in Supplementary File 2.

2.8. Statistical Analysis. The results were expressed as mean ± SD or mean ± SEM (for gene expressions), and differences were considered significant when $P < 0.05$, $P < 0.10$ were set as the trend of difference. Treatment means were compared by independent sample $T$-test with SPSS 11.0 for windows.

3. Results

3.1. Production Performance of Offspring Chicks. As shown in Table 1, adding GEN into the diet of LBB hens significantly increased the body weight gain of chicks during day 1-21. However, feed intake and the feed gain ratio were not significantly different between the CON and GEN groups. The breast muscle rate and liver index were significantly higher in the GEN group than the CON group ($P < 0.05$), while the abdominal fat percentage of the chick was decreased after GEN supplementation for LBB hens ($P = 0.094$). The GEN treatment made no significantly effects on the heart index of chicks.

3.2. Lipid Metabolic Indexes and GEN Content in Offspring Chicks. As shown in Table 2, serum CHOL ($P < 0.01$) and LDL levels of chicks from the GEN group ($P < 0.05$) were decreased compared to the CON group, while the serum HDL level was increased after GEN treatment. The levels of GEN in the livers of 21-day-old chicks were not significantly different between the CON and GEN groups (Table 3).
Table 1: Growth performance of 21-day-old broiler chicks.

| Index                | CON    | GEN    | P value |
|----------------------|--------|--------|---------|
| Body weight gain (kg)| 0.791 ± 0.020 | 0.824 ± 0.009 | 0.001 |
| Feed intake (kg)     | 1.08 ± 0.05  | 1.11 ± 0.04  | 0.114 |
| Feed gain ratio      | 1.37 ± 0.04  | 1.34 ± 0.04  | 0.287 |
| Breast muscle rate (%)| 21.3 ± 0.87  | 23.18 ± 1.49 | 0.031 |
| Abdomen fat rate (%) | 0.017 ± 0.001| 0.014 ± 0.003| 0.094 |
| Heart index (%)      | 0.52 ± 0.06  | 0.53 ± 0.29  | 0.843 |
| Liver index (%)      | 2.24 ± 0.33  | 2.73 ± 0.25  | 0.013 |

CON: control group; GEN: GEN-treated group. Growth performance indicators, including body weight gain, feed intake, and feed gain ratio, were calculated by the average value of each cage, n = 8 cages. The organ indexes include breast muscle and abdominal fat percentage and heart and liver indexes. Organ index = organ weight/body weight. The average value of organ indexes of two chickens in one cage represents the whole replicate, n = 8 cages.

Table 2: Hepatic antioxidative capability of 21-day-old broiler chicks.

| Index               | CON    | GEN    | P value |
|---------------------|--------|--------|---------|
| T-AOC (U/mg prot)   | 0.60 ± 0.21 | 0.95 ± 0.24 | 0.014 |
| MDA (mmol/mg prot)  | 0.94 ± 0.42 | 0.69 ± 0.16 | 0.012 |
| CAT (U/mg prot)     | 14.77 ± 3.05 | 14.92 ± 5.31 | 0.949 |
| T-SOD (U/mg prot)   | 295 ± 48  | 373 ± 82  | 0.038 |
| GSH-Px (U/mg prot)  | 22.81 ± 3.15 | 25.92 ± 4.37 | 0.123 |

CON: control group; GEN: GEN-treated group. T-AOC (total antioxidant capacity); MDA (malondialdehyde); CAT (catalase); T-SOD (total superoxide dismutase); GSH-Px (glutathione peroxidase), n = 8.

Adding GEN to the diet of LBB hens reduced the LCFA (Table 3, P < 0.05), cholesterol (P < 0.05), and triglyceride (P < 0.063) in the chick livers. Remarkably, GEN treatment decreased the levels of C14:0, C21:0, C20:5n3, and C22:6n3 in the chick livers (P < 0.05). Meanwhile, C16:0, C17:0, and C18:1n9c content were presented with a decreased trend after GEN supplementation (P < 0.05). Hepatic n-6 family LCFAAs (P = 0.089), monounsaturated LCFAAs (MUFAs, P = 0.090), and polyunsaturated LCFAAs (PUFAs, P = 0.078) of the GEN group tended to reduce compared with the CON group. However, the content of C20:4n6 (arachidonic acid) and C23:0 was significantly higher in the GEN group than in the CON group (P < 0.05).

3.3. Hepatic Antioxidant Indexes of Chicks. As shown in Table 3, adding GEN to the diet of LBB hens increased T-SOD activity (P < 0.05) and decreased MDA content (P < 0.05) in the liver of chicks. Hepatic T-AOC of the GEN group was significantly higher than the CON group (P < 0.05). The activities of CAT and GSH-Px in the liver of the GEN group were not significantly different from the CON group.

3.4. The Hormone Levels in the Chick Serum. As we can see from Table 4, the levels of T3 and T4 in serum were not
3. RNA Sequencing (RNA-Seq) Data of the Chick Livers. After analyzing the hepatic RNA-Seq data from 21-day-old chicks, we obtained a total of 642,089,356 clean reads, averaging 80,261,169 clean reads (73,869,122 to 87,132,554) per sample (Table S4). The average Q30 value was 91.26%. The number of reads from all samples aligned to Gallus gallus (assembly Gallus_gallus 5.0, https://www.ncbi.nlm.nih.gov/genome/111?genome_assembly_id) was 260,603, significantly different between the two groups. The level of GH in the serum of the GEN group was presented with an average alignment ratio of 83.755%. The number of DEGs discussed in the current paper were shown in Table S6. In addition, adding GEN into the LBB hen diet significantly promoted offspring lipid transport, cholesterol metabolic processes, and glycolysis (Table 4). We further conducted the adjusted analysis on DEG clusters using Bonferroni statistical overrepresentation tests. As shown in Table 5, 10 DEGs clustered with glycolgen metabolism (GO:0005975) and 108 DEGs clustered with lipid metabolism (GO:0006629). In the fatty acid metabolic process (Figure 2(c)), we obtained clustered terms, including the acyl-CoA metabolic process (GO:0006637; ENSGALG 00000013848, mevalonate kinase; ACSM3, medium-chain acyl-CoA synthetase; ACOT9, acyl-CoA thioesterase 9; MVD, mevalonate diphosphate decarboxylase; HMGCS2, hydroxymethylglutaryl-CoA synthase; FAR1, fatty acyl-CoA reductase 1; and PMVK, phosphomevalonate kinase), fatty acid β-oxidation (GO:0006663s; ACADL, long-chain specific acyl-CoA dehydrogenase; ECHDC1, ethylmalonyl-CoA decarboxylase; CRYL1, Lambda-crystallin homolog; HADHA, trifunctional enzyme subunit alpha; EC11, enoyl-CoA delta isomerase 1; E1TA, electron transfer flavoprotein subunit alpha; ACOX2, acyl-CoA oxidase; and ACAT2, acyl-CoA acyltransferase), and fatty acid biosynthetic process (GO:0006633; PTGES, prostaglandin E synthase; ALOX5AP, arachidonate 5-lipoxygenase-activating protein; and ENSGALG00000011450, leukotriene c4 synthase). The transcriptional levels of the abovementioned genes were significantly upregulated in the livers of GEN-treated LBB hen offspring compared with those of the CON hen offspring (Table S6). As shown in the KEGG pathway analysis (Figure 3), GEN supplementation for LBB hens significantly upregulated the transcriptional level of VLDL in the liver of offspring and activated the PPAR signaling pathway (PPARD, peroxisome proliferator-activated receptor delta 3.21[7.33; SLC27A1, solute carrier family 27 member 1, 4.96[7.81; ACOX2, acyl-CoA oxidase 2, 80.38[130.25; HMGCS2, hydroxymethylglutaryl-CoA synthase, 567.82[920.71; APOA5, apolipoprotein A-V, 213.7[355.11; PLTP, phospholipid transfer protein, 2.19[5.31; ACSV, long-chain acyl-CoA synthetase, 96.26[173.67; PLIN1, perilipin-1, 0.67[1.37; ACSBG1, 0.35[0.73; FABP3, fatty acid-binding protein, 2.19[5.69; CYP7A1, cholesterol 7alpha-monooxygenase, 24.28[71.42; ANGPTL4, angio poietin-like 4, 24.56[93.49; ACADL, long-chain-acyl-CoA dehydrogenase, 339.21[531.46; ACACB2, acyl-CoA synthetase bubblegum family member 2, 49.74[208.31; VLDL, very low-density lipoprotein, 2.23[4.89; UBE2R2, ubiquitin-conjugating enzyme E2 R2, 12.82[19.80; and DBI, acyl-CoA binding protein, 557.62[721.32). From the overrepresentation test (Table 5), DEGs were clustered into the insulin receptor signaling pathway (IRS2, insulin receptor substrate 2 [0.60; SOCS1, suppressor of cytokine signaling 1 [1.51; PTPN2, tyrosine-protein phosphatase nonreceptor type 2 [1.51; CCND3, G1S-specific cyclin-D3[1.52; ZNF106, zinc finger protein 106[1.53; AKT1, RAC serine/threonine-protein kinase [1.54; IRS1, insulin receptor substrate 1 [1.56; PHP, PH-interacting protein [1.56; PRKCD, protein kinase C delta[1.58; NCOA5, nuclear receptor coactivator 5[1.59; PIK3R3, phosphoinositide-3-kinase regulatory subunit 3 [1.67; STXBP4, syntaxin-binding protein 4[1.68; SIK2, serine/threonine-protein kinase SIK2[1.71; TSC1, tuberous

| Treatment | T3 (ng/ml) | T4 (ng/ml) | GH (pg/ml) |
|-----------|------------|------------|------------|
| CON       | 1.04 ± 0.36 | 55.81 ± 5.93 | 1.09 ± 0.09 |
| GEN       | 1.00 ± 0.20 | 51.89 ± 5.92 | 1.19 ± 0.10 |
| P value   | 0.753      | 0.128      | 0.067      |

T3/T4 (triiodothyronine); GH (growth hormone); E2 (estrogen); n = 8.

3.5. Confirmation of the Accuracy of the RNA-Seq Transcriptome Data by Quantitative Real-Time PCR. To confirm the accuracy of the RNA-Seq transcriptome data, we randomly selected 17 DEGs. The expression levels of selected genes were quantified using qRT-PCR, and the results were consistent with the findings obtained by RNA-Seq (Figures 1(a) and 1(b)). The results suggested that RNA-Seq reliably identified DEGs in the chicken liver transcriptome.

3.7. DEG Analysis by cuffdiff Software and the PANTHER Classification System. As shown in Table S5, 3915 DEGs were obtained between the CON and GEN groups by cuffdiff software analysis. The expressions and fold change of DEGs discussed in the current paper were shown in Table S6. We further identified 3092 DEGs using PANTHER analysis. As shown in Figure 2(a), 4550 biological processes were obtained by DEG cluster analysis. There were 1377, 1050, 504, and 332 genes, respectively, clustering into the cell process, metabolic process, reproduction, developmental process, and response to stimulus categories. As shown in Figure 2(b), 22 DEGs, including PKM, pyruvate kinase; GANC, glucosidase alpha; RANBP1, Ran-binding protein 1; PGD, 6-phosphogluconate dehydrogenase; TKT, transketolase; PGM5, phosphoglucomutase-like protein 5; HK1/HK2, hexokinase; RPE, ribulose-phosphate 3-epimerase; LDHB, L-lactate dehydrogenase; SYNJ2, synaptotagmin 2; PGM2, phosphoglucomutase; HKDC1, hexokinase domain containing 1; RGN, glucuronolactonase; and PGM2L1, glucose-1,6-bisphosphate synthase clustered with the monosaccharide metabolic process (GO:0005996). Furthermore, DEGs (LDHB, L-lactate dehydrogenase; CS, citrate synthase; ID H3A, isoanichrome dehydrogenase 3 (NAD+) alpha) clustered with the tricarboxylic acid cycle.
sclerosis 1 |1.74; PID1, phosphotyrosine interaction domain containing 1 |1.79; CAV2, caveolin 2 |1.80; NUCKS1, nuclear casein kinase and cyclin-dependent kinase substrate 1 |1.81; SMARCC1, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily C|1.89; SOGA1, suppressor of glucose autophagy associated 1 |2.01; and PIK3R2, phosphoinositide-3-kinase regulatory subunit 2 |2.09) and insulin-like growth factor signaling pathway (EIF2AK3, eukaryotic translation initiation factor 2-alpha kinase 3 |1.52; AR, aldehyde reductase|1.52; AKT1, serine/threonine-protein kinase|1.54; GIGYF2, GRB10 interacting GYF protein 2|1.54; IRS1, insulin receptor substrate 1 |1.56; PLCB1, phospholipase C beta 1|1.56; IGFBP1, insulin-like growth factor-binding protein 1|1.59; ATXN1, ataxin 1|1.80; CILP, cartilage intermediate layer protein|1.90; and CDH3, cadherin 3|2.20). GEN treatment promoted the cellular amino acid (AA) metabolic process and developmental growth, as well as inhibited the apoptotic process. Among these genes, IGF1 and IGFBP1 expressions were significantly upregulated. Additionally, GEN supplementation activated the MAPK signaling pathway, I-kappaB kinase/NF-kappaB cascade, and response to interferon-gamma in offspring.

Regarding molecular function (Figure 2(d)), DEGs were mainly clustered with catalytic activity (GO:0016209; MGST3, microsomal glutathione S-transferase 3; MGST2, microsomal glutathione S-transferase 2; SOD3, superoxide dismutase 3; PER2, period circadian regulator 2; PDXN, peroxidase; ALOX5AP, arachidonate 5-lipoxygenase-activating protein; LTC4SL, leukotriene C4 synthase; MT1, metallothionein 1; and MT4, metallothionein 4).

### 3.8. Histone Modification Profile Using RNA-Seq Analysis

The effect of maternal nutrition on the development and metabolism of offspring is regulated by epigenetic modification. Thus, we conducted GO analysis on histone modification using genes that were differentially expressed between the CON and GEN groups. As we can see from Table S7, adding GEN into the diet of LBB hens significantly influenced methylation (GO:0032259) and histone lysine methylation (GO:0034968) in the offspring chick livers. GEN treatment altered histone H3-K36 methylation, histone H3-K36 trimethylation, and histone H3-K36 demethylation and upregulated the expression of specific methyltransferases (SETD2, histone-lysine N-methyltransferase; IWS1, transcription factor SPN1; and NSD1, nuclear receptor binding SET domain protein 1). Additionally, DEGs were clustered into histone H3-K4 trimethylation and histone lysine dimethylation. GEN
supplementation upregulated histone acetyltransferase (EP300, E1A binding protein p300; EP400, E1A binding protein p400; and MYST2, lysine acetyltransferase 7) expression and promoted histone acetylation, including histone H4-K8 and histone H4-K12 acetylation in chick liver. GEN supplementation for LBB hens also promoted mRNA transcription from the RNA polymerase II promoter and histone monoubiquitination.

3.9. Maternal GEN Supplementation Affects Histone Trimethylation and Acetylation at the Promoter Region of PPARD in the Liver of Offspring Chicks
3.9.1. Chromatin Fragmentation Result. Under electrophoretic conditions (2% agarose gel electrophoresis; 140 V; 25 min), most chromatin fragments are between 100 and 500 bp (Figure S3).

3.9.2. ChIP-qPCR Primer Information. We chose two promoter sites on the PPARδ gene (PPARD; chr26:4048020+4048156 and chr26:4047862+4047963) to detect histone modification status. Primer information is shown in Table S3.

3.9.3. ChIP-qPCR Results. As shown in Figure 4, two primers for the promoter of PPARD gene were used to amplify the DNA enriched by ChIP. We compared DNA enriched by the four antibodies (H3K4me3, H3K36me3, H4K8ace, and H4K12ace) with that by IgG. The results showed the relative amount of enriched DNA precipitated by H3K36me3 and H4K12ac at the P1 and P2 site of the PPARD promoter from the liver of the GEN group was higher than the CON group. However, the relative amount of enriched DNA precipitated by H3K4me3 and H4K8ace at both the P1 and P2 site of PPARD was not significantly different between the two groups. GEN supplementation for LBB hens induced H3K36me3 and H4K12ac modifications at the promoter (chr26:4048020+4048156 and chr26:4047862+4047963) of PPARD in the liver of offspring chicks.

4. Discussion

4.1. Effect of Maternal GEN Supplementation on the Growth Performance of Offspring Chicks. ISFs reportedly promote the growth and reproductive performance of livestock [36, 37]. It is suggested that dietary GEN 20 to 80 mg/kg not only improves growth performance but also beneficially affects immunological responses in broiler chicks [38]. Transgenerational effects involve epigenetic changes and phenotypic changes induced by maternal and environmental factors, leading to developmental changes in offspring [39]. However, the effect of dietary GEN supplementation for LLB hens on the growth of offspring is still unknown. In this experiment, LLB hens during the late egg-laying period were selected as an experimental model, in which egg-laying rate and egg quality decreased rapidly. Adding 40 mg/kg GEN to the diet of LBB hens significantly increased the body weight gain of chicks during the early growth stage, which is consistent with the increased chicken embryo body size parameters that we previously reported (Figure S2). Protein metabolism in broiler chicks is more robust than fat metabolism during the early growth period. We found that DEGs from the liver transcriptome were clustered into the developmental process and cellular AA metabolic process. Meanwhile, GEN treatment upregulated the insulin signaling pathway and glycolytic process in the chick livers, which promote glucose transformation into AAs.

Table 5: Results of the PANTHER classification system statistical overrepresentation test of Cuffdiff results (CON vs. GEN).

| PANTHER GO-Slim biological process | Gallus gallus REFLIST (15782) | Observed | Expected | Over/under | Fold enrichment | P value |
|-----------------------------------|--------------------------------|----------|----------|------------|-----------------|---------|
| Metabolic process                 | 4935                           | 1050     | 822.28   | +          | 1.28            | 7.99E-17 |
| Lipid metabolic process           | 544                            | 108      | 90.64    | +          | 1.19            | 5.91E-02 |
| Fatty acid metabolic process      | 151                            | 41       | 25.16    | +          | 1.63            | 6.92E-03 |
| Fatty acid biosynthetic process   | 39                             | 8        | 6.5      | +          | 1.23            | 5.34E-01 |
| Unsaturated fatty acid biosynthetic process | 6 | 3 | 1 | + | 3 | 1.26E-01 |
| Fatty acid beta-oxidation          | 21                             | 8        | 3.5      | +          | 2.29            | 3.71E-02 |
| Acyl-CoA metabolic process        | 27                             | 7        | 4.5      | +          | 1.56            | 2.21E-01 |
| Lipid transport                   | 61                             | 17       | 10.16    | +          | 1.67            | 3.23E-02 |
| Cholesterol metabolic process     | 25                             | 6        | 4.17     | +          | 1.44            | 4.37E-01 |
| Glycogen metabolic process        | 30                             | 10       | 5        | +          | 2               | 3.68E-02 |
| Cellular ketone body metabolic process | 11 | 3 | 4.02 | + | 1.75 | 6.03E-02 |
| Bile acid metabolic process       | 2                              | 1        | 0.06     | +          | 16.99           | 8.33E-02 |
| Cellular amino acid metabolic process | 222 | 57 | 36.99 | + | 1.54 | 4.41E-03 |
| Developmental growth              | 446                            | 81       | 200.37   | +          | 1.51            | 6.31E-04 |
| Glycolysis                         | 31                             | 9        | 5.17     | +          | 1.74            | 4.69E-02 |
| Negative regulation of apoptotic process | 101 | 31 | 16.83 | + | 1.84 | 3.98E-03 |
| MAPK signaling pathway            | 297                            | 76       | 49.49    | +          | 1.54            | 1.26E-03 |
| Insulin-like growth factor receptor signaling pathway | 27 | 10 | 51.23 | + | 1.66 | 1.21E-03 |
| PPAR signaling pathway            | 51                             | 14       | 9.35     | +          | 1.49            | 2.14E-02 |
| Insulin receptor signaling pathway | 82                             | 20       | 8.68     | +          | 1.79            | 2.91E-03 |
| I-kappaB kinase/NF-kappaB cascade  | 41                             | 8        | 0.83     | +          | 1.17            | 4.82E-01 |
| Response to interferon-gamma      | 43                             | 10       | 7.16     | +          | 1.40            | 3.26E-01 |
GEN can reportedly increase the plasma GH concentration in ewes during the infusion period [40]. In the present study, supplementing LBB hens with 40 mg/kg GEN increased the serum GH level in chicks, following with the increased IGF-1 and IGFBP1 mRNA expressions in the liver. The liver can produce IGFs under the action of GH. IGFs have potent mitogenic effects on target organs through receptor- and IGFBP-mediated regulation [41]. Additionally, IGFs can promote DNA synthesis, proliferation, and differentiation, as well as inhibit the apoptotic process [42]. Accordingly, PANTHER overrepresentation tests revealed that GEN treatment inhibited the apoptotic process in chicks. IGF-1 reportedly promotes postnatal development and cell proliferation by activating the PI3K/Akt signaling pathway [43–45]. In our study, maternal GEN supplementation activated the IGFs signaling pathway in the chick livers, with increased PI3K (PIK3CD, PIK3R5) and AKT (AKT1, AKT2) mRNA expression. In summary, we describe here for the first time that adding GEN into the diet of LBB hens can promote the developmental growth of offspring chicks by activating the GH-IGF1-PI3K/Akt signaling pathway.

### 4.2. Effect of Maternal GEN on the Antioxidative Capability of Chicks

Broilers are a type of poultry with a high growth speed and strong metabolic rate, which produces a large number of free radicals. Superoxide anion radical (O2-) induces lipid peroxidation, which can damage the structure and function of biofilms, leading to metabolic disorders in the body [46]. It is reported that ISFs can inhibit the oxidation of active ingredients in the body by binding to specific LDL sites [47]. Therefore, studying the effect of maternal GEN on the antioxidant capacity of offspring is greatly meaningful. In the current study, adding GEN into the diet of LBB hens significantly improved T-AOC and inhibited lipid peroxidation in the chick livers. A SOD-CAT-GSH-Px-based enzyme system in the body plays an important role in the antioxidant processes. Maternal GEN supplementation significantly increased T-SOD activity in the chick livers. PANTHER cluster analysis also showed that GEN treatment affected peroxidase activity (GO:0004601) in the chick liver with upregulated MnSOD (SOD3) mRNA expression. GEN reportedly exerts antioxidant capability by binding to ESRs, leading to the rapid activation of the ERK1/2 and NFκB signaling pathways and a delayed upregulation of MnSOD gene expression in MCF-7 cells [48]. Similarly, maternal GEN supplementation upregulated the transcriptional level of ESR1 in the liver of chicks and activated the I-kappaB kinase/NF-kappaB cascade. Therefore, maternal GEN supplementation might upregulate MnSOD mRNA expression by activating ESR-NFκB in the offspring. Metallothionein has approximately 10,000 times the ROS-scavenging capacity of SOD [49]. In the present experiment, supplementing LBB

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**Figure 3:** DEGs clustered in the PPAR signaling pathway. Genes marked in red were upregulated (P adjusted < 0.05, |fold change| > 1.5). Genes marked in pink were upregulated (P adjusted < 0.05, 1.0 < |fold change| < 1.5).
hens with GEN significantly increased MT1 and MT4 mRNA expression in the chick livers. This finding is consistent with the report that GEN can increase metallothionein expression in Caco-2 cells [50]. Our study provides the first evidence that maternal GEN supplementation can increase SOD and MT mRNA expression, as well as the antioxidant capacity of chicks. Therefore, the effect of maternal GEN supplementation might protect next-generation chicks against oxidative stress, which can improve the growth performance.

4.3. Effect of Maternal GEN Supplementation Alters Lipid Metabolism and Enzyme Transcription in Broiler Chicks. Antenatal phytoestrogen exposure can reduce the risk of metabolic syndrome in offspring [51, 52]. GEN supplementation increased VLDL transcription in chicks. The main function of VLDL is to transport hepatic TGs to peripheral tissues, which can decrease the level of TGs in the serum and liver. Furthermore, cluster analysis indicated that GEN treatment affected fatty acid biosynthesis, acyl-CoA metabolic process, and lipid transport in chicks. Further detection showed that GEN supplementation significantly decreased LCFA levels in the liver. GEN reportedly increases energy expenditure and fatty acid β-oxidation in hepatocytes [53]. Cuffdiff analysis clearly showed that maternal GEN supplementation significantly upregulated the transcriptional levels of β-oxidation key enzymes, including ACADL, ACADSB, ACAD8 (ester acyl-CoA dehydrogenase), HADHA (hydroxyacyl-CoA dehydrogenase), ECHDC (ethylmalonyl-CoA decarboxylase), ETFA (electron transfer flavoprotein subunit alpha), ACOX2 (acyl-CoA oxidase), ACAT2 (acetoacetyl-CoA thiolase), and ACOT (acyetyl-CoA C-acetyltransferase). Therefore, GEN supplementation decreased the LCSFA and PUFA levels in the chick livers. ACAT catalyses acyl-CoA hydrolysis into free fatty acid (FFA) and CoA, which promotes fatty acid β-oxidation and carboxylic acid transport [54, 55]. Meanwhile, we found that GEN supplementation significantly increased the transcriptional levels of arachidonate 5-lipoxigenase-activating...

![Figure 4: Relative enrichment level/input of qPCR products using the promoter site primer. IgG is the negative control; the four histone modification sites included histone H3 lysine 4 trimethylation (H3K4me3), histone H3 lysine 36 trimethylation (H3K36me3), histone H4 lysine 8 acetylation (H4K8ac), and histone H4 lysine 12 (H4K12ac). The results are expressed as the means ± SD. (a) P1 site (chr26:4048020+4048156); (b) P2 site (chr26:4047862+4047963).](image)
protein, C-fos and Egr-1, as well as the AA levels in the chick livers. Studies have shown that AAs can upregulate the expression of C-fos and Egr-1, which promote cell growth and organ development [56]. This regulation might be one of the pathways by which maternal GEN supplementation promotes progeny growth.

4.4. Effects of Dietary GEN Supplementation for LLB Hens on the Cholesterol Metabolism in Offspring Chicks. Fatty acids in hepatic mitochondria are decomposed into acetyl-CoA, which then are mainly converted into energy, cholesterol, ketone bodies, and new fatty acids. Adding GEN into the diet of LBB hens significantly upregulated the transcriptional levels of HMGCoA lyase (HMGL), HMGCoA synthase (HMGCS2), and 3-oxoacid CoA-transferase (OXCT1) in the chick liver, which promoted ketone body formation. GEN treatment upregulated progesterone kinase (MVD) and phosphomevalonate kinase (PMVK) mRNA expression, which are the key enzymes in cholesterol synthesis. In addition, the cholesterol 7α-hydroxylase (CYP7A1) transcription level in the liver and the serum HDL level of chicks were increased after GEN supplementation in LBB hens, but the serum LDL level was decreased. It is well-established that LDL carries cholesterol from the liver to other tissues; HDL sends cholesterol back to the liver; then, cholesterol is metabolized into bile acids or drained from the intestine [57]. CYP7A1 is a key enzyme that catalyses bile acid synthesis from cholesterol. Thus, GEN supplementation for LBB hens promoted the conversion of cholesterol into bile acid in chicks. Accordingly, we found that cholesterol in the liver and serum of chicks were both reduced after GEN treatment. Studies demonstrate that GEN can increase the serum HDLC level and decrease the cholesterol and LDLC level in hyperlipidaemic patients [58], which need further studies to explore the mechanism. It was reasonable to conclude that maternal GEN supplementation promoted cholesterol transportation and decomposition in offspring chicks.

4.5. Effects of Dietary GEN Supplementation for LBB Hens on Hepatic PPARδ Expression of Offspring Chicks and Its Mechanism. PPARs play important roles in the regulation of fatty acid metabolism. GEN can promote fatty acid catabolism by activating PPARα and PPARγ [59]. In this study, GEN treatment did not significantly affect PPARα or PPARγ expression in offspring birds but upregulated the expression of PPARδ and target genes. PPARδ is mainly involved in the cellular lipid metabolism, which promotes reverse cholesterol transport and oxidative decomposition of lipid [60]. Furthermore, ACOT, which is strongly activated by PPARs, is able to terminate fatty acid synthesis [61]. Therefore, we speculated that maternal GEN supplementation has the ability to regulate the network that decreases lipid levels in offspring through PPARδ. We further explored the mechanism by which maternal GEN affects PPARδ expression in offspring. First, we doubt that the dietary GEN supplementing for LBB hens can deposit into the chick liver, which serve as the ligand to activate the PPARδ signaling pathway directly. However, the contents of GEN in the chick livers were not different between the CON and GEN group. Interestingly, studies have shown that prostaglandins, eicosanoids, and retinoic acid are natural ligands of PPARδ [62]. Our study showed that hepatic AA levels are significantly higher in GEN-treated offspring chicks compared with the CON group, which might activate the PPARδ signaling pathway.

Epigenetic regulations, including DNA methylation, histone modification, chromatin remodeling, and RNA interference, can be inherited from breeders to offspring, which affect the offspring’s development [63, 64]. Cluster analysis indicated that GEN supplementation for LLB hens affects histone lysine methylation and histone acetylation in the chick liver, including H3-K36 methylation, H3-K4 methylation, H4-K8 acetylation, and H4-K12 acetylation. Therefore, we speculated that maternal GEN might upregulate PPARδ expression in the chick livers through histone methylation and acetylation. ChiP-qPCR data verified that H3-K36me3 and H4-K12 acetylation were increased at P1 and P2 sites of PPARδ gene promoter in the chick liver after GEN supplementation for LBB hens. H3K36 methylation participates in the regulation of gene transcription. It is reported that large amounts of H3K36me3 are enriched at the 3′-end of the active transcriptional gene in zebrafish somatic cells [65]. In the current experiment, GEN treatment increased the transcriptional levels of H3-K36 methylation-related genes and methyltransferase (ASH 1L, SETD3, NSD1, and SMYD2), as well as SET domain-containing protein 2 (SETD2) in the chick livers. SETD2 is an H3K36 trimethyltransferase that associates with hyperphosphorylated RNA polymerase II and transcriptional elongation [66], which is consistent with the upregulated mRNA transcription from the RNA polymerase II promoter in offspring chicks. NSD1 is a SET domain histone methyltransferase that primarily methylates nucleosomal H3K36. NSD1 depletion can reportedly decrease the levels of H3K36me2 and H3K36me3 [67]. Furthermore, the transcriptional level of Iws1 is higher in the chick liver of the GEN group compared with the CON group. It is reported that Iws1:Sppt6:CTD can promote cotranscriptional mRNA synthesis and histone H3K36 methylation regulated by Setd2 [68]. Therefore, GEN supplementation for LBB hens might enhance the epigenetic modification of histone H3-K36 trimethylation at the promoter region of PPARδ in offspring chicks by upregulating Iws1 and methyltransferase expression. Histone methylation, acetylation, and DNA methylation usually have a synergistic effect on gene transcriptional regulation. Transcriptional activation markers such as H3K4me3, H3K36me3, histone acetylation, and hypomethylation often exist in chromatin fragments where gene transcription is active at the same time [69]. The MYST family acts as lysine acetyltransferases, involving in gene expression and DNA damage repair [70]. In this experiment, the different expressed MYST 2 was clustered with H4-K12 acetylation. Thus, GEN supplementation for LBB hens might promote the epigenetic modification of histone H4-K12 acetylation at the promoter region of PPARδ in the chick livers by upregulating MYST 2 expression. Histone methylation and demethylation, as well as acetylation and deacetylation, are dynamically balanced processes. We also
found GEN treatment influenced both H3-K4 demethylation and histone monoubiquitination processes in offspring chicks. Considering the ChIP-qPCR data, GEN supplementing for LBB hens might induce H3-K4 methylation or H4-K8 acetylation at other gene sites of offspring chicks.

5. Conclusion

Adding GEN into the diet of LBB hens activated the GH-IGFs-PI3K/Akt pathway in offspring chicks, which increased the body weight gain and organ indexes. Maternal GEN supplementation upregulated MnSOD and MT expression in chick livers, which improved the antioxidative capability. Interestingly, GEN treatment activated H3-K36 trimethylation at the promoter of PPARD through Iws1 and methyltransferases, as well as induced H4-K12 acetylation at the promoter region of PPARD through MYST2, which activated the PPARδ signaling pathway in the chick liver. Furthermore, GEN supplementation for LBB hens enhanced fatty acid beta-oxidation, acyl-CoA metabolic process, lipid transport, and cholesterol metabolism in the chick livers. In conclusion, supplementing GEN for LBB hens alters the fatty acid metabolism and growth performance of offspring chicks by epigenetic modification.

Abbreviations

CON: Control
ROS: Reactive oxygen species
T3: Triiodothyronine
T4: Tetraiodothyronine
GH: Growth hormone
E2: Estradiol
DEGs: Differentially expressed genes
BWG: Body weight gain
FI: Feed intake
FCR: Feed conversion ratio
GEN: Genistein
ISF: Isoflavone
MT: Metallothionein
T-AOC: Total antioxidant capacity
MDA: Malondialdehyde
CAT: Catalase
T-SOD: Total superoxide dismutase
GSH-Px: Glutathione peroxidase
LB: Laying broiler breeder
ChIP: Chromatin immunoprecipitation
HDL/LDL: High/low density lipoprotein
LCFAs: Long-chain fatty acids
RPKM: Per kilobase per million
MUFAs: Monounsaturated LFCAs.

Data Availability

The data of growth performance, antioxidative capability, biochemical index, LCFA content, PANTHER statistical overrepresentation test and GO-Slim analysis, relative RNA expression of genes, DEGs clustered in the PPAR signaling pathway, and enrichment level of ChIP-qPCR used to support the findings of this study are included within the article. The data of diet composition, primers, RNA-Seq statistics, different expressed genes, functional cluster analysis of histone modifications, embryonic body sizes, and fragment size after chromatin fragmentation used to support the findings of this study are included within the supplementary information files.

Conflicts of Interest

The authors have no financial or personal conflicts of interest to declare.

Authors’ Contributions

H.F. and Z.L. contributed equally to this work. All authors read and approved the final version of the manuscript.

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Supplementary Materials

Supplementary 1. A photographic abstract.
Supplementary 2. Assay procedure of chromatin ChIP-qPCR.
Supplementary 3. Table S1: diet composition and nutrient levels.
Supplementary 4. Table S2: primers used for quantitative real-time PCR analysis.
Supplementary 5. Table S3: ChIP-qPCR primer information.
Supplementary 6. Table S4: RNA-Seq statistics of samples used in the experiment.
Supplementary 7. Table 5: the different expressed genes between the CON and GEN groups.
Supplementary 8. Table S6: results of differential expression gene (CON vs. GEN) analysis using Cuffdiff software.
Supplementary 9. Table 7: the functional cluster analysis of histone modifications using DEGs.
Supplementary 10. Figure S1: proportion of the exonic and intronic regions aligned to the reference genome.
Supplementary 11. Figure S2: the effects of maternal GEN on the body sizes of embryos at days 11, 13, 15, 17, and 19.
Supplementary 12. Figure S3: fragment size after chromatin fragmentation.

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