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Effects of Dietary Vitamin E on Intramuscular Fat Deposition and Transcriptome Profile of the Pectoral Muscle of Broilers

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Vitamin E is an essential micronutrient for animals. The aim of this study was to determine the effect of vitamin E on intramuscular fat (IMF) deposition and the transcriptome profile of the pectoral muscle in broiler chickens. Arbor Acres chickens were divided into five treatment groups fed a basal diet supplemented with 0, 20, 50, 75, and 100 IU/kg dietary DL-α-tocopheryl acetate (vitamin E), respectively. Body weight, carcass performance, and IMF content were recorded. Transcriptome profiles of the pectoral muscles of 35-day-old chickens in the control and treatment groups (100 IU/kg of vitamin E) were obtained by RNA sequencing. The results showed that diets supplemented with 100 IU/kg of vitamin E significantly increased IMF deposition in chickens on day 35. In total, 159 differentially expressed genes (DEGs), including 57 up-regulated and 102 down-regulated genes, were identified in the treatment (100 IU/kg vitamin E) group compared to the control group. These DEGs were significantly enriched in 13 Gene Ontology terms involved in muscle development and lipid metabolism; three signaling pathways, including the mitogen-activated protein kinase and FoxO signaling pathways, which play key roles in muscular and lipid metabolism; 28 biofunctional categories associated with skeletal and muscular system development; 17 lipid metabolism functional categories; and three lipid metabolism and muscle development-related networks. The DEGs, pathways, functional categories, and networks identified in this study provide new insights into the regulatory roles of vitamin E on IMF deposition in broilers. Therefore, diets supplemented with 100 IU/kg of vitamin E will be more beneficial to broiler production.

Key words: broiler, differentially expressed genes, intramuscular fat, transcriptome profile, vitamin E

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Introduction

The intramuscular fat (IMF) content is an important determinant of the quality, tenderness, moisture content, and flavor of chicken meat (Li *et al.*, 2009; Sun *et al.*, 2013). IMF deposition is dependent on the balance among lipid synthesis, transport, uptake, and subsequent metabolism, which involve various genes and pathways (Qiu *et al.*, 2017). As the heritability of IMF content is relatively low (h^2 = 0.07–0.20) (Zhao *et al.*, 2006), regulation of IMF deposition is mainly achieved through nutritional supplementation (Hocquette *et al.*, 2010).

Vitamin E is an essential, non-toxic, fat-soluble micronutrient in both humans and animals. There are eight structurally related forms of vitamin E: α-, β-, γ-, and δ-tocopherol, and α-, β-, γ-, and δ-tocotrienol, with α-tocopherol being among the most active forms (González-Calvo *et al.*, 2017). The antioxidant and non-antioxidant properties of vitamin E largely contribute to livestock performance and product quality (Idamokoro *et al.*, 2020). In general, vitamin E supplementation has shown no impact on the growth performance or meat yield of livestock (Wang *et al.*, 2020). In animals, α-tocopherol plays an important role in the regulation of lipid metabolism (Sun et al., 2015). In addition to its antioxidant properties, α-tocopherol is a cell-signaling

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molecule that alters the expression of genes involved in lipid metabolism in the bovine longissimus thoracis muscle (Ladeira *et al.*, 2020). However, methionine/α-tocopherol reportedly has limited effects on the crude fat content and intramuscular fatty acid profile of the longissimus lumborum muscle in Hanwoo cattle (Barido *et al.*, 2020). In chickens, dietary supplementation with vitamin E can reduce the severity of "wooden breast" myopathy and promote breast meat quality without adversely affecting the growth performance and meat yield (Wang *et al.*, 2020). In addition, dietary supplementation with vitamin E decreased the abdominal fat content of broilers (Li *et al.*, 2009; Zaboli *et al*., 2013), which was also observed in response to heat stress (Habibian *et al.*, 2016). Dietary supplementation with vitamin E (200 mg/kg) for 120 days significantly increased the IMF content in the breast and thigh muscles of female Beijing-you chickens (Li *et al.*, 2009). However, another study reported that supplementation of vitamin E at 200 mg/kg for a period of 63 days had no significant effect on the IMF content of the breast and thigh muscles of an experimental line of male Polish chickens (Zdanowska-Sąsiadek *et al.*, 2016). In addition, previous studies have reported that vitamin E had no effect on the IMF content and fatty acid profile (Bellés *et al.*, 2018) of lamb and chicken meat (Rebolé *et al.*, 2006). Therefore, it remains controversial whether dietary vitamin E can increase the IMF content of broilers, which may be related to differences in the breed of chicken and the vitamin E supplementation level used across studies.

Our previous transcriptome analyses showed that vitamin E influences the abdominal fat content of broilers (Zhang *et al.*, 2020; Zhang *et al.*, 2021). Hence, the aim of the present study was to investigate the regulatory roles of vitamin E in IMF deposition, and to identify key genes, signaling pathways, functional categories, and genetic networks associated with IMF deposition in broilers through RNA-sequencing.

Materials and methods

Animals, experimental design, and diets

The study protocol was approved by the Ethics Committee of Longyan University (No. LY2022003X; Longyan, Fujian province, China). One-day-old healthy female Arbor Acres chicks (n = 240) with a mean body weight (BW) of 42.35 ± 1.92 g were randomly allocated to one of five dietary treatment groups. Each treatment group consisted of six replicates, with each replicate consisting of eight chicks. The composition of the basal diet adapted from previous studies (Zhang *et al.*, 2020; Zhang *et al.*, 2021) is shown in **Supplementary Table S1.** The vitamin E concentrations in the diets administered on days 0–21 and 21–35 were 82.4 and 90.1 mg/kg, respectively. The chickens in the five treatment groups were fed the basal diet supplemented with 0, 20, 50, 75, and 100 IU/kg of dietary DL-α-tocopheryl acetate (Xinchang Hebao Biotechnology Co., Ltd., Shaoxing, Zhejiang province, China) over a trial period of 35 days. All broilers were raised in stair-step cages with *ad libitum* access to food and water. A 23-h light and 1-h dark lighting schedule was provided. A standard temperature program was used with 35°C for the first 3 days and then reduced by 3°C weekly until reaching 23–25°C. Chickens were administered the Newcastle disease and infectious bronchitis combined vaccine at 7 and 21 days, and were administered a vaccine of infectious bursal disease virus at 14 days.

BW, carcass performance, and IMF content

At the end of each week, one broiler from each replicate was randomly selected, fasted for 12 h, weighed, and killed via stunning and exsanguination. The weights of the bilateral breast muscles, including the pectoralis major and minor (BrW), deboned thigh (ThW), and liver (LiW), were measured as described previously (Sun *et al.*, 2013). The eviscerated yields (BrW, ThW, and LiW) as percentages of the live weight (BrP, ThP, and LiP) were calculated. The pectoralis major muscle was sampled and stored in liquid nitrogen until further use.

The percentage of IMF of the breast was measured as described in detail in an earlier report (Cui *et al.*, 2012b). In brief, following elimination of obvious fat, 2.0 g of each sample was thoroughly minced and dried in two 10–12-h stages at 65°C and 105°C, respectively, and then cooled in a desiccator for at least 30 min. The IMF content of the breast samples was measured using the Soxhlet extraction method with anhydrous ether as the solvent and is expressed as the percentage of dry tissue weight.

mRNA library construction and sequencing

Total RNA was isolated from three pectoralis major tissue samples each from the control group and high-dose vitamin E supplement group (100 IU/kg), which were matched for similar weight, using the RNAsimple Total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China), in accordance with the manufacturer's instructions. The integrity and concentration of RNA were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) to meet the quality requirements for sequencing. mRNA was enriched from total RNA samples using oligo (DT) magnetic beads (Invitrogen Corporation, Carlsbad, CA, USA) and then treated with Ambion DNase I (Life Technologies, Carlsbad, CA, USA). First-strand cDNA was synthesized from purified mRNA and fragmented with an RNA fragmentation kit (Ambion, Foster City, CA, USA), followed by second-strand cDNA synthesis according to the manufacturer's instructions. The quality of the cDNA library was assessed using an Agilent 2100 Bioanalyzer and StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Finally, qualified cDNA libraries were sequenced using an Illumina HiSeq 2500 system (Illumina, Inc., San Diego, CA, USA).

RNA-sequencing data process and analysis

The raw reads mapped to each gene were quantified using easyRNASeq software (Delhomme *et al.*, 2012). Clean data were acquired from quality raw data reads using SOAPnuke software (Chen *et al.*, 2018). Reads with contaminating adapters, more than 5% unknown bases, and low-quality bases (fewer than 15 bases, >20%) were removed. All clean reads were mapped onto a reference chicken genome (GRCg6a/galGal6) using TopHat v2.0.12 software (https://ccb.jhu.edu/software/tophat/-downloads/) with default parameters (Kim and Salzberg, 2011; Ghosh and Chan, 2016). Genes were annotated using Ensembl v67

(Larsson *et al.*, 2005).

Gene expression levels were calculated using RSEM v1.2.12 (Li and Dewey, 2011). A heatmap of all expressed genes was plotted using the pheatmap package (Kolde and Kolde, 2015) of R v3.4.3.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

GO analysis of differentially expressed genes (DEGs) was performed using Blast2GO (Conesa *et al.*, 2005) with a false discovery rate-adjusted *p*-value of < 0.05. The online KEGG Automatic Annotation Server (Moriya *et al.*, 2007) was used to assign KEGG pathways to the individual genes. In all tests, *p*-values were calculated using the Benjamini-corrected modified Fisher's exact test, and a corrected *p*-value < 0.05 was considered statistically significant.

Bio-function and network enrichment analysis

Ingenuity Pathway Analysis (IPA) (Krämer *et al.*, 2014) was performed to determine the functions and networks of the DEGs. The biological functions of the DEGs were based on "disease and function analysis" of the core analysis. Networks were obtained based on gene connectivity.

Validation of RNA-sequencing results by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed to verify the transcription levels of 14 randomly selected DEGs (seven up-regulated: *FHL2*, *HPGD*, *AMHR2*, *SESN1*, *WNT16*, *ZBTB16*, and *FOXO3*; seven down-regulated: *CSF1*, *FOS*, *LOC107056699*, *MMP9*, *SELE*, *SOCS3*, and *VCAM1*). The chicken housekeeping gene β-actin was used as a reference gene for quantification. All primer sequences were designed using Primer Premier software (version 5.0; Premier Biosoft, Palo Alto, CA, USA). The primer sequences for qPCR used in this study are listed in **Supplementary Table S2**.

Total RNA was extracted from the pectoralis major tissue samples using the RNAsimple Total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China). First-strand cDNA was synthesized from 2 μg total RNA using a reverse transcription kit (Promega, Madison, WI, USA). The mRNA levels of selected genes were analyzed using Power SYBR®Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany). qPCR was performed using an ABI 7500 Real-Time Detection System (Applied Biosystems).

The amplification reaction volume was 20 μL, containing 10 μL of 2× PCR Master Mix, 100 ng cDNA, 0.5 μL of each primer (10 μmol), and 8.0 μL ddH_2O. To ensure nearly 100% PCR efficiency between the gene of interest and the reference gene (β-actin), primers and cDNA concentrations were optimized as needed. PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 32 s. The comparative cycle threshold (CT) method (Livak *et al*., 2001) was used to calculate relative fold-changes in gene expression from triplicate biological repeat samples, as $2^{-\Delta\Delta CT}$.

Statistical analysis

Differences in BW, carcass performance, and IMF content of the pectoralis major muscle among the groups were identified using the GLM procedure (SAS V8, SAS Institute, Cary, NC, USA). The results are expressed as mean ± standard deviation. Multiple comparisons were performed using the Tukey *post-hoc* test. Statistical significance was set at $p \leq 0.05$. Statistical analyses of gene expression levels between the control and treatment groups were performed using Student's *t*-test (IBM SPSS Statistics 19.0, SPSS Inc., Chicago, IL, USA).

Results

BW, carcass performance, and IMF content

There were no significant differences in BW and carcass performance (BrW, BrP, ThW, ThP, LiW, and LiP) between the control and vitamin E treatment groups on days 7, 14, 21, and 35 (**Supplementary Table S3**). At day 35, the IMF content of the breast muscle was significantly higher in the treatment group that received 100 IU/kg of vitamin E than that in the control and other treatment groups ($p < 0.05$) (**Figure 1** and **Supplementary Table S4**).

Mapped reads and DEGs

The clean mapped reads were 40.17–71.71 Mb in length with percentages of 73.82%–80.23% (**Supplementary Table S5**), which met the sequencing requirements. The clean RNA-sequencing data from this study were deposited in the NCBI BioProject (Accession No. PRJNA744211). Of the 159 DEGs identified between the control group and the group treated with vitamin E at 100 IU/kg, 57 were up-regulated and 102 were down-regulated (**Figure 2**; **Supplementary Table S6**). The top 10 up-regulated and 10 down-regulated DEGs are listed in **Table 1**.

GO and KEGG pathway analyses

The DEGs were significantly enriched in 236 GO terms (corrected $p < 0.05$) (**Supplementary Table S7**), including 13 terms related to skeletal muscle cell differentiation (corrected $p = 1.27 \times 10^{-3}$), muscle structure development (corrected $p = 2.82 \times 10^{-3}$), and cellular lipid metabolic processes (corrected $p = 3.26 \times 10^{-3}$) (**Table 2**). In addition, 16 DEGs were significantly enriched in three signaling pathways: the AGE-RAGE signaling pathway in diabetic complications, mitogen-activated protein kinase (MAPK) signaling pathway, and FoxO signaling pathway (corrected $p < 0.05$) (**Table 3**).

Function and network analyses

In total, 118 DEGs were mapped to the IPA database. The functional analysis results showed that the DEGs were classified into two main categories: (i) molecular and cellular functions and (ii) physiological system development and functions (**Table 4**). In addition, 31 DEGs were classified into 17 lipid metabolism functions, and 46 DEGs were classified into 28 skeletal and muscular system development and functions (**Supplementary Table S8** and **Table S9**). These functional categories were mainly related to lipid concentration, synthesis, secretion, and oxidation (**Supplementary Table S8**), as well as muscle differentiation and morphology (**Supplementary Table S9**). Ten networks were identified in the IPA database (**Supplementary Table S10** and **Figures S1–S10**). Of these, networks 1, 2, and 8 were associated with lipid metabolism and muscle development (**Supplementary Figures S1–S3**).

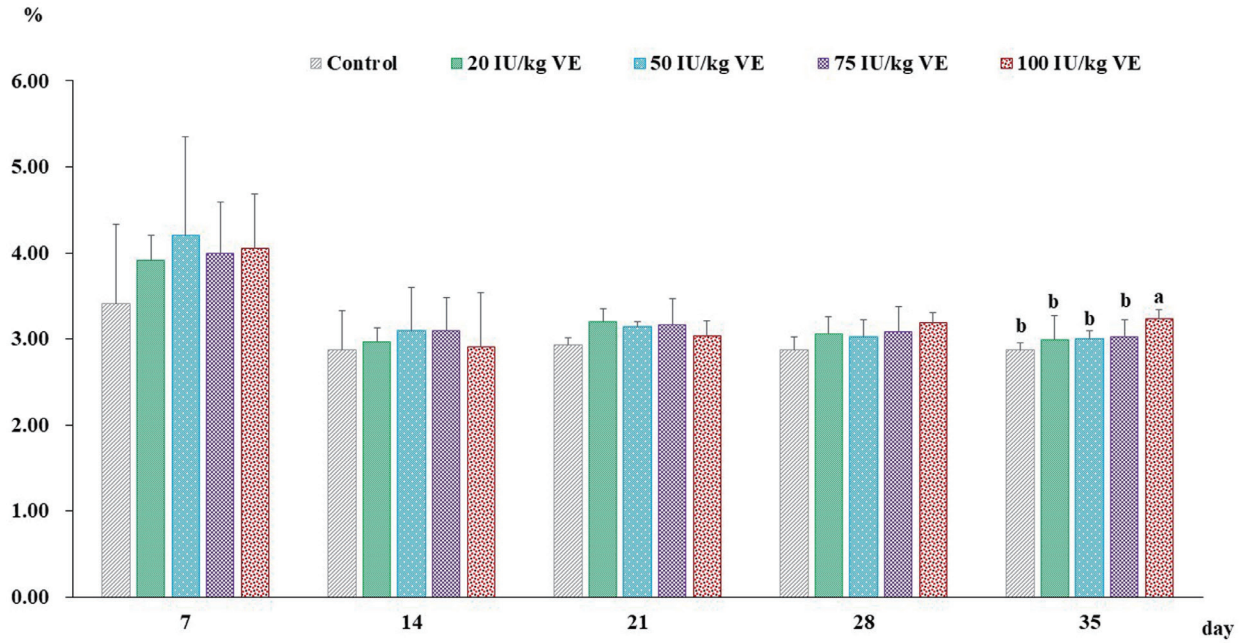

Fig. 1. **Effect of dietary vitamin E on IMF deposition in the breast muscle of broilers (six birds per group). Different lowercase superscript letters indicate significant differences ($p < 0.05$).**

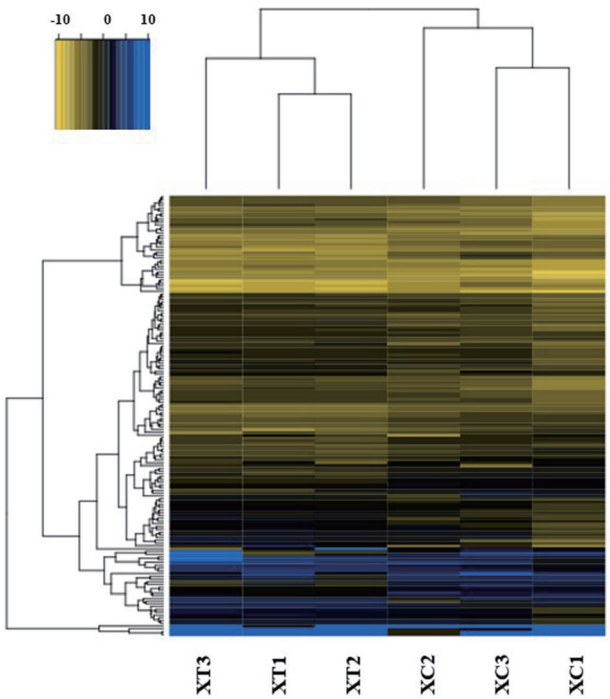

Fig. 2. **Heatmap of differentially expressed genes (DEGs) of each sample: XC1, XC2, and XC3 from control groups; XT1, XT2, and XT3 from vitamin E supplementary groups. Blue indicates a high expression level and yellow indicates a low expression level.**

qPCR validation

The qPCR analysis of 14 selected genes (*FHL2*, *HPGD*, *AMHR2*, *SESN1*, *WNT16*, *ZBTB16*, *FOXO3*, *CSF1*, *FOS*, *LOC107056699*, *MMP9*, *SELE*, *SOCS3*, and *VCAM1*) was consistent with the RNA-sequencing analysis results (**Supplementary Table S11**), indicating that the RNA-sequencing results in this study were valid.

Discussion

Vitamin E supplementation of corn and soybean meal reportedly has no effect on the growth performance of broilers (Pompeu *et al.*, 2018), although moderate and high dietary levels of vitamin E may influence other functions (Leshchinsky and Klasing, 2001). In practice, a high-dose vitamin E diet is provided to improve the immune capacity of birds (Boa-Amponsem *et al.*, 2000; Khan *et al.*, 2012), and their resistance to heat stress (Niu *et al.*, 2009) and oxidative stress (Gao *et al.*, 2010). The results of our study showed that vitamin E supplementation had no effect on the BW or carcass performance of broilers. In particular, high dietary levels of vitamin E (approximately 20-fold greater than stated in "Nutrient Requirements of Poultry: Ninth Revised Edition," 1994) can improve IMF deposition in the pectoral muscle of broilers by altering the transcriptome. However, vitamin E supplementation of less than 100 IU/kg had no effect on IMF deposition during the 35-day trial, indicating that vitamin E regulation of fat metabolism might be dependent on a relatively high

Table 1. **Top 10 up-regulated and top 10 down-regulated differentially expressed genes.**

Gene	Log_2(XT/XC)	*p*	Corrected *p*	Up/down-regulated
KCTD20	0.58	9.01×10^{-14}	2.36×10^{-10}	up
SLC43A2	0.43	4.37×10^{-13}	1.00×10^{-9}	up
LOC427491	0.25	2.72×10^{-12}	4.75×10^{-9}	up
SESN1	0.50	2.64×10^{-8}	2.21×10^{-5}	up
CD36	0.83	2.89×10^{-8}	2.30×10^{-5}	up
LOC107050812	0.35	6.44×10^{-8}	4.65×10^{-5}	up
LOC107055521	0.23	7.87×10^{-8}	5.31×10^{-5}	up
BG2	0.29	8.38×10^{-8}	5.48×10^{-5}	up
TRIM63	0.60	1.57×10^{-7}	9.38×10^{-5}	up
IGSF10	0.56	6.27×10^{-7}	2.98×10^{-4}	up
SELE	–0.23	1.12×10^{-29}	2.34×10^{-25}	down
CNN1	–0.44	6.46×10^{-24}	6.77×10^{-20}	down
KLHL40	–0.47	3.03×10^{-22}	2.11×10^{-18}	down
ATF3	–0.39	3.57×10^{-18}	1.87×10^{-14}	down
MYH1A	–0.42	8.52×10^{-17}	3.57×10^{-13}	down
PNOC	–0.34	4.07×10^{-15}	1.42×10^{-11}	down
TNC	–0.61	3.45×10^{-14}	1.03×10^{-10}	down
LOC100858388	–0.25	4.79×10^{-13}	1.00×10^{-9}	down
RFWD2	–0.62	1.94×10^{-12}	3.69×10^{-9}	down
EDA2R	–0.48	9.65×10^{-12}	1.55×10^{-8}	down

Note: *ATF3*, activating transcription factor 3; *BG2*, intestinal zipper protein; *CD36*, CD36 molecule; *CNN1*, calponin 1; *EDA2R*, ectodysplasin A2 receptor; *IGSF10*, immunoglobulin superfamily member 10; *KCTD20*, potassium channel tetramerization domain containing 20; *KLHL40*, kelch like family member 40; *LOC100858388*, 78 kDa glucose-regulated protein-like; *LOC107050812*, Ig mu chain C region-like; *LOC107055521*, uncharacterized LOC107055521; *LOC427491*, C2 calcium dependent domain containing 4A; *MYH1A*, myosin, heavy chain 1A, skeletal muscle (similar to human myosin, heavy chain 1, skeletal muscle, adult); *PNOC*, prepronociceptin; *RFWD2*, ring finger and WD repeat domain 2; *SELE*, selectin E; *SESN1*, sestrin 1; *SLC43A2*, solute carrier family 43 member 2; *TNC*, tenascin C; *TRIM63*, tripartite motif containing 6; XT, treatment group; XC, control group.

dosage and an additive effect.

Vitamin E is considered to regulate IMF deposition by acting as a regulatory factor in the transcriptional control of genes related to lipid metabolism in the muscle (González-Calvo *et al.*, 2015). IMF is distributed in the epimysium, perimysium, and endomysium of the muscle tissues (Cui *et al.*, 2018). Triglycerides, phospholipids, and cholesterol are important constituents of the lipid fraction in muscle cells (Liu *et al.*, 2019). The genes that regulate muscle and fat metabolism also regulate IMF deposition. The present study identified 159 DEGs in the breast muscle of broilers between the control and vitamin E supplementation groups. Six genes (*ASB2*, *HOPX*, *FOS*, *FHL2*, *HLF*, and *MAFF*) were significantly enriched in seven GO terms associated with muscle cell differentiation and muscle tissue development, whereas seven genes (*ST3GAL1*, *CETP*, *PDK4*, *CYR61*, *AGTR1*, *UCP3*, and *HPGD*) were significantly enriched in six GO terms related to lipid metabolism. Thus, these DEGs may play key roles in IMF deposition. For example, ankyrin repeat and SOCS box-containing (ASB)2, which is encoded by *ASB2*, is a member of ASB protein family that negatively regulates muscle mass (Davey *et al.*, 2016). Moreover, cholesteryl ester transfer protein, which is encoded by *CETP*, is involved in the transfer of cholesteryl esters from high-density lipoproteins to other lipoproteins (Zhang *et al.*, 2012). Pathway analysis was used to explore the regulatory signaling pathways underlying the vitamin E regulation of IMF deposition in the pectoralis major muscle of broilers. The results identified three pathways: the AGE-RAGE signaling pathway, which is associated with diabetes complications, and the MAPK and FoxO signaling pathways, which play important roles in IMF deposition and muscle development. Eight DEGs (*IGF2*, *FOS*, *AMHR2*, *CSF1*, *MAP3K4*, *JUND*, *DUSP5*, and *DUSP10*) associated with the MAPK signaling pathway regulate processes involving tight junctions, focal adhesions, and the actin cytoskeleton. A previous study reported that cell junctions might interact with the MAPK signaling pathway, which is associated with lipid metabolism and thus influences the deposition of IMF (Cui *et al.*, 2012a). Four DEGs (*FOXO3*, *AMHR2*, *FOXO6*, and *FBXO32*) are constituents of the FoxO signaling pathway, which is reportedly involved in development of the chicken breast muscle (Li *et al.*, 2019). However, further investigation is needed to elucidate the molecular mechanisms by which these genes and pathways regulate IMF deposition.

Bio-functional analysis identified 55 DEGs, including 31 involved in 17 lipid metabolism functional categories and 46

Table 2. **Muscle development and lipid metabolism-related Gene Ontology (GO) analysis results.**

Term	ID	Corrected *p*	Genes
Cellular lipid metabolic process	GO:0044255	3.26×10^{-3}	*ST3GAL1*, *CETP*, *PDK4*, *CYR61*, *AGTR1*, *UCP3*, *HPGD*
Lipid metabolic process	GO:0006629	1.18×10^{-2}	*ST3GAL1*, *CETP*, *PDK4*, *CYR61*, *AGTR1*, *UCP3*, *HPGD*
Regulation of lipid metabolic process	GO:0019216	4.29×10^{-2}	*AGTR1*, *CYR61*, *PDK4*
Sphingolipid biosynthetic process	GO:0030148	4.61×10^{-2}	*ST3GAL1*, *CYR61*
Fatty acid metabolic process	GO:0006631	4.65×10^{-2}	*UCP3*, *PDK4*, *HPGD*
Regulation of lipase activity	GO:0060191	4.73×10^{-2}	*AGTR1*, *CYR61*
Skeletal muscle cell differentiation	GO:0035914	1.27×10^{-3}	*ASB2*, *HLF*, *MAFF*, *FOS*
Muscle structure development	GO:0061061	2.82×10^{-3}	*ASB2*, *HOPX*, *FOS*, *FHL2*, *HLF*, *MAFF*
Striated muscle tissue development	GO:0014706	3.73×10^{-3}	*FHL2*, *HLF*, *ASB2*, *MAFF*, *FOS*
Muscle tissue development	GO:0060537	4.22×10^{-3}	*FHL2*, *HLF*, *ASB2*, *MAFF*, *FOS*
Skeletal muscle tissue development	GO:0007519	4.50×10^{-3}	*ASB2*, *HLF*, *MAFF*, *FOS*
Skeletal muscle organ development	GO:0060538	5.28×10^{-3}	*ASB2*, *HLF*, *MAFF*, *FOS*
Muscle organ development	GO:0007517	1.59×10^{-2}	*ASB2*, *HLF*, *MAFF*, *FOS*

Note: *AGTR1*, angiotensin II receptor type 1; *ASB2*, ankyrin repeat and SOCS box containing 2; *CETP*, cholesteryl ester transfer protein; *CYR61*, cysteine-rich angiogenic inducer 61; *FHL2*, four and a half LIM domains 2; *FOS*, Fos proto-oncogene, AP-1 transcription factor subunit; *HLF*, HLF, PAR bZIP transcription factor; *HOPX*, HOP homeobox; *HPGD*, hydroxyprostaglandin dehydrogenase 15-(NAD); *MAFF*, MAF bZIP transcription factor F; *PDK4*, pyruvate dehydrogenase kinase 4; *ST3GAL1,* ST3 beta-galactoside alpha-2,3-sialyltransferase 1; *UCP3*, uncoupling protein 3.

Table 3. **Pathway analysis results.**

Pathway	ID	*p*	Corrected *p*	Genes
AGE-RAGE signaling pathway in diabetic complications	gga04933	8.22×10^{-6}	6.84×10^{-4}	*AMHR2*, *PIM1*, *SELE*, *EGR1*, *VCAM1*, *AGTR1*,
MAPK signaling pathway	gga04010	5.83×10^{-5}	2.37×10^{-3}	*IGF2*, *FOS*, *AMHR2*, *CSF1*, *MAP3K4*, JUND, *DUSP5*, *DUSP10*
FoxO signaling pathway	gga04068	3.66×10^{-3}	3.67×10^{-2}	*FOXO3*, *AMHR2*, *FOXO6*, *FBXO32*

Note: *AGTR1*, angiotensin II receptor type 1; *AMHR2*, anti-Mullerian hormone receptor type 2; *CSF1*, colony stimulating factor 1; *DUSP10*, dual specificity phosphatase 10; *DUSP5*, dual specificity phosphatase 5; *EGR1*, early growth response 1; *FBXO32*, F-box protein 32; *FOS*, Fos proto-oncogene, AP-1 transcription factor subunit; *FOXO3*, forkhead box O3; *FOXO6*, forkhead box O6; *IGF2*, insulin-like growth factor 2; *JUND*, JunD proto-oncogene, AP-1 transcription factor subunit; *MAP3K4*, mitogen-activated protein kinase kinase kinase 4; *PIM1*, Pim-1 proto-oncogene, serine/threonine kinase; SELE, selectin E; *VCAM1*, vascular cell adhesion molecule 1; MAPK, mitogen-activated protein kinase.

Table 4. **Top biological functions of the differentially expressed genes.**

Biological functions	*p* range	# Molecules
Molecular and Cellular Functions		
Cellular Development	3.72×10^{-4}–2.43×10^{-9}	57
Cellular Growth and Proliferation	3.72×10^{-4}–6.27×10^{-9}	52
Cell Death and Survival	3.83×10^{-4}–1.87×10^{-8}	57
Cell Morphology	2.50×10^{-4}–1.90×10^{-8}	36
Lipid Metabolism	2.98×10^{-4}–1.67×10^{-7}	31
Physiological System Development and Functions		
Tissue Morphology	3.94×10^{-4}–1.38×10^{-10}	58
Cardiovascular System Development and Function	3.92×10^{-4}–1.49×10^{-10}	44
Organismal Development	3.75×10^{-4}–3.70×10^{-10}	58
Organ Morphology	3.92×10^{-4}–2.19×10^{-9}	37
Skeletal and Muscular System Development and Function	3.92×10^{-4}–2.43×10^{-9}	46

involved in 28 skeletal and muscular system development and functional categories. Among these, 22 DEGs (*ADA*, *AGTR1*, *ATF3*, *CCN1*, *CD36*, *CSF1*, *EGR1*, *FOS*, *FOXO3*, *HLF*, *IGF2*, *MAFF*, *MMP9*, *NR4A3*, *PDK4*, *PDPN*, *RDH10*, *RRAD*, *SOCS3*, *VCAM1*, *WNT16*, and *ZBTB16*) were classified in both functional categories, indicating that these DEGs participate in the regulation of fat and muscle metabolism. IPA networks are based on the Ingenuity Knowledge Base, which includes information on biomolecules and their associated relationships (Reyes-Gibby *et al.*, 2017). Of the 10 networks identified in this study, three were related to lipid metabolism and muscle development, indicating that these genes and networks are potentially directly or indirectly associated with IMF deposition. For example, *EGR1*, which was located at the center of the radial plots of the two networks, was downregulated in the muscle tissue in response to dietary supplementation with vitamin E and was in the center of a network related to lipid metabolism. *EGR1* encodes early growth response 1, a nuclear protein that acts as a transcriptional regulator that inhibits adipocyte differentiation (Boyle *et al.*, 2009), while *EGR1* loss-of-function promotes adipocyte differentiation (Bléher *et al.*, 2020). Thus, a high dose of vitamin E likely inhibited *EGR1* expression, thereby relieving the inhibitory effect of *EGR1* on adipocyte differentiation in the breast muscle tissue of broilers and increasing IMF deposition, although this hypothesis needs further confirmation. Overall, the functions of the networks identified in this study significantly influenced IMF deposition.

The strength of our study is the confirmation that high vitamin E supplementation can increase the IMF content in the breast muscle of broilers, along with the identification of DEGs, signaling pathways, and networks in the pectoralis major muscle of broilers with dietary supplementation of vitamin E, which primarily explains the regulatory role of vitamin E on IMF deposition. However, our study has potential limitations. Whether vitamin E can increase the IMF content in the breast muscle by increasing adipocyte proliferation and differentiation needs to be further confirmed at the (pre)adipocyte level. Moreover, the IMF content does not always improve meat quality. Excessive fat deposition leads to the formation of white-striping breast in broilers (Zambonelli *et al.*, 2016). The relationship between IMF content in the breast muscle and white-striping breast requires further study.

In summary, IMF disposition was increased by dietary supplementation with vitamin E at 100 UI/kg, and 159 DEGs were identified in the pectoralis major muscle of broilers with vitamin E supplementation. Of these DEGs, IPA results indicated that 55 were enriched in lipid metabolism, skeletal and muscular system development, and other related functional categories. Furthermore, we found that the MAPK and FoxO signaling pathways play key roles in muscle and lipid metabolism. Some DEGs identified in this study were associated with three lipid metabolism- and muscle development-related networks. The DEGs, pathways, functional categories, and networks identified in this study provide insights into the regulatory role of vitamin E in IMF deposition in broilers.

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Authors' contributions

Y.S. conceived, designed, and supervised the study. M.Z., W.L., Q.W., Y.L., D.C., and L.L. performed the animal trials. Y.S. and M.Z. performed the statistical analyses and wrote the manuscript. All authors contributed to manuscript preparation and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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