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Effect of myristic acid supplementation on triglyceride synthesis and related genes in the pectoral muscles of broiler chickens

Xiaojing Liu,[†,*] Yanke Wang,[†] Yidong Wang,[†] Huanxian Cui,[†] Guiping Zhao,[†] Yuming Guo,[*] and Jie Wen[†,1]

[†]*State Key Laboratory of Animal Biotech Breeding, State Key Laboratory of Animal Nutrition and Feeding, Institute of Animal Science, Chinese Academy of Agricultural Sciences (CAAS), Beijing 100193, China; and* [*]*State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China*

ABSTRACT Fatty acids (**FAs**) can serve as energy for poultry, maintain normal cell structure and function, and support a healthy immune system. Although the addition of polyunsaturated fatty acids (**PUFAs**) to the diet has been extensively studied and reported, the mechanism of action of saturated fatty acids (**SFAs**) remains to be elucidated. We investigated the effect of 0.04% dietary myristic acid (**MA**) on slaughter performance, lipid components, tissue FAs, and the transcriptome profile in chickens. The results showed that dietary MA had no effect on slaughter performance (body weight, carcass weight, eviscerated weight, and pectoral muscle weight) ($P > 0.05$). Dietary MA enrichment increased MA ($P < 0.001$) and triglycerides (**TGs**) ($P < 0.01$) levels in the pectoral muscle. The levels of palmitic acid, linoleic acid (**LA**), arachidonic acid (**AA**), SFAs, monounsaturated fatty acids (**MUFAs**), and PUFAs were significantly higher ($P < 0.01$) in the MA supplementation group compared to the control group. However, there were no significant differences in the ratios of PUFA/SFA and n6/omega-3 (**n3**) between the two groups. The MA content was positively correlated with the contents of palmitic acid, LA, linolenic acid (**ALA**), n3, n6, SFAs, and unsaturated fatty acids (**UFA**). *DHCR24*, which is known to be involved in steroid metabolism and cholesterol biosynthesis pathways, was found to be a significantly lower in the MA supplementation group compared to the control group ($P < 0.05$, log2(fold change) = -0.85). Five overlapping co-expressed genes were identified at the intersection between the differential expressed genes and Weighted Gene Co-expression Network Analysis-derived hub genes associated with MA phenotype, namely *BHLHE40, MSL1, PLAGL1, SRSF4*, and ENSGALG00000026875. For the TG phenotype, a total of 28 genes were identified, including *CHKA, KLF5, TGIF1*, etc. Both sets included the gene *PLAGL1*, which has a negative correlation with the levels of MA and TG. This study provides valuable information to further understand the regulation of gene expression patterns by dietary supplementation with MA and examines at the molecular level the phenotypic changes induced by supplementation with MA.

Key words: myristic acid, chicken, pectoral muscle, regulatory gene, PLAGL1

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INTRODUCTION

Chicken is a major source of animal protein in the human diet. Consumption preferences change as people's living standards improve, consumers are increasingly choosing chicken products that are low in fat, high in protein, high in unsaturated fat content, and have a delicate taste (Huo et al., 2022). The demand for Chinese local chicken pectoral muscle meat is also growing due to its rich polyunsaturated fatty acid (**PUFAs**) content, which directly influences the tenderness, flavor, and taste of chicken breast meat.[1]

FAs, as essential components of triglycerides (**TGs**) and phospholipids (**PLIPs**), play a critical role in the formation of intramuscular fat (**IMF**). The dietary supplementation of FAs can impact fat deposition in broilers. On the one hand, unsaturated FAs (**UFAs**) can promote the proliferation and differentiation of fat

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[1]Corresponding author: wenjie@caas.cn

[1]The genome sequence data reported in this article is being uploaded to the genome sequence file of the BIG Data Center of the Beijing Institute of Genomics, Chinese Academy of Sciences, and is publicly available from http://bigd.big.ac.cn (CRA0016343).

cells, leading to increased adipose tissue (Wongsuthavas et al., 2008). On the other hand, they influence the metabolic pathways of FA synthesis and degradation, thereby regulating the extent of fat deposition. Some studies have shown that using feed rich in UFA can enhance the taste and storage characteristics of meat (Orczewska-Dudek and Pietras, 2019). It can increase the fluidity of muscle cell membranes, thus improving meat tenderness and taste (Risha et al., 2021). Additionally, it can alter the composition and distribution of FAs in meat, thereby influencing its flavor and texture.

Moderate dietary supplementation with UFAs can increase fat deposition, improve meat quality, and enhance the immune function and antioxidant capacity of broilers (Al-Khalaifah, 2020). Numerous studies have investigated the effects of UFAs on poultry from various aspects, including performance, meat quality, serum biochemical indices, and muscle FA composition (Kim et al., 2016; Wang et al., 2022). The dietary supplementation of UFAs has been found to reduce blood TG levels and improve the overall health status of poultry (Shahid et al., 2019). A study evaluating the feeding duration of UFAs revealed that the inclusion of flaxseed oil had no detrimental effects on the performance and meat quality of ducks, but improved the FA composition of muscles (Zhang et al., 2023). Moreover, incorporating UFAs into poultry diets not only alters the content of muscle FAs, but also increases the levels of omega-3 (**n3**) PUFAs in eggs and decreases the ratio of omega-6 (**n6**) to n3 (Ribeiro et al., 2013).

The effects of dietary supplementation of PUFAs on lipid metabolism in poultry have been well studied, but the effects of saturated FAs (**SFAs**) on FAs in poultry remain unclear. Myristic acid (**MA**) is a 14-carbon FA, whose content in animal tissues is usually very low, generally accounting for less than 1% of the total FAs (Saraswathi et al., 2022). Studies in humans and mice have shown that a diet rich in MA can cause hypercholesterolemia (Temme et al., 1997). In addition, MA is involved in protein myristoylation, a post-translational modification that alters various signaling pathways, thereby regulating cell function (Udenwobele et al., 2017). Experiments involving the addition of MA to the diet have shown certain effects on rat lipid metabolism. For instance, a study has reported that treatment with MA significantly increased intracellular TG content in rat hepatocellular carcinoma cells while reducing TG secretion (Kummrow et al., 2002). Rioux et al. (2005) found significant changes in the proportion of SFAs, monounsaturated FAs (**MUFAs**), and PUFAs in the liver, plasma, and adipocytes of rats fed with different doses of MA (0.2%−1.2% of energy level) (Rioux et al., 2005). These findings suggest that MA can influence FA composition. However, whether MA can regulate lipid content and FA composition in chickens has not been reported.

In recent years, with the rapid development of high-throughput sequencing technology, transcriptomics has emerged as a comprehensive, dynamic, and high-throughput research technique (Tian et al., 2022; Fan et al., 2023; Zhai et al., 2023). Studies using transcriptomics have successfully identified key genes involved in lipid metabolism and FA synthesis in poultry and established gene co-expression networks. Therefore, to elucidate the mechanism and regulatory pathways mediating the effects of exogenous MA on muscle lipids in chickens, this study evaluated the effects of an 8-week MA diet on performance, muscle lipid composition, and FA composition in chickens. Additionally, transcriptome sequencing was performed on individuals in the supplementation group and the control group, and the changes in gene expression patterns and action pathways after supplementation with MA were analyzed by identifying differentially expressed genes (**DEGs**), constructing the gene co-expression network by Weighted Gene Co-expression Network Analysis (**WGCNA**) and exploring related genes. In summary, the main purpose of this study is to provide a theoretical basis and a reference for MA metabolism in muscle from the perspective of gene expression data and diet composition, as well as further elucidate the mechanism of action and regulatory pathways of FAs and improve the production efficiency and chicken meat quality.

MATERIALS AND METHODS

Experimental Design and Sample Collection

The Jinling hua chickens used in this study were obtained from Guangxi Jinling Agricultural and Livestock Group Co., Ltd. (Guangxi, China). A total of 100 one-day old healthy female Jinlinghua chickens were selected and randomly divided into 2 groups with each group comprising 5 replicates of 10 chickens per replicate. All experimental protocols were approved by the Science Research Department (which is in charge of animal welfare issues) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (Beijing, China) (No. IAS2019-21). The MA content in the different treatment groups was as follows: the control group (0% MA), the 56-d group (0.04% MA) (Kiarie et al., 2024). The MA was procured from Guangzhou Insighter Biotechnology Co., Ltd, Guangzhou, China.

The experimental chickens were raised in cages with a feeding period from 1 to 56 days of age. The chickens were maintained according to the core breeding management process of the facility, allowing ad libitum access to feed and water. The chicken coop had good ventilation, with suitable environmental temperature, humidity, and lighting conditions, along with routine immunization and disease prevention measures. The experimental diet was formulated by the company and the formulation and nutritional composition of the experimental diet are shown in Table 1. All individuals were 56 days old to slaughter. Pectoral muscle samples were collected and stored at −80℃ until RNA isolation. The remaining pectoralis major muscle tissues were collected and stored at −20℃ until the measurement of FAs, TGs, PLIPs, and total cholesterol (**TCHO**) contents.

Table 1. Composition and main characteristics of feed.

Item	Contents	
	1 to 21 days old	21 to 56 days old
Ingredients (%)		
Corn	63.9	67.35
Soybean meal	30.73	28
Soybean oil	0.8	0.4
Limestone	1.8	1.9
Dicalcium phosphate	1.35	1
Salt	0.3	0.4
Lysine	0.22	0.22
Methionine	0.2	0.17
Choline chloride	0.2	0.14
Premix[1]	0.5	0.42
Nutrient levels[2]		
Metabolic energy/(MJ/kg)	2900	2800
Crude protein	19	17.5
Lysine	1.0	0.9
Methionine	0.5	0.48
Cysteine	0.81	0.78
Threonine	0.75	0.67
Tryptophan	0.24	0.22
Calcium	1.15	1.1
Available phosphorus	0.47	0.45

[1]The premix provides per kilogram: VA 13,200 IU, VD3 5,000 IU, VE 60 IU, VK3 8.0 mg, thiamine 4.0 mg, riboflavin 12.0 mg, pyridoxine 12.0 mg, cobalamin 0.4 mg, niacin 80 mg, pantothenic acid 24 mg, folic acid 2.0 mg, biotin 3.0 mg, iron 70.0 mg, copper 8.5 mg, zinc 45.0 mg, manganese 60.0 mg, iodine 0.85 mg, selenium 0.20 mg, cobalt 0.25 mg. The nutritional components are calculated values based on actual additions.

[2]The nutrient levels were calculated values.

Measurement and Calculation of Slaughter Performance Traits

The measurement and calculation of slaughter performance traits were conducted by the agricultural industry standard "NYIT 823-2020 Poultry Production Performance Terminology and Measurement Calculation Methods." Fifteen chickens were randomly selected for slaughter in the MA supplementation group and the control group. After slaughter, the slaughter weight was recorded. Pre-slaughter weight: The weight of the chickens is measured after deprived of feed for 12 hours. Slaughter weight: The weight of the chicken after bleeding, feathers, the horny layer of the feet, the beak and the intestines were removed. Eviscerated weight: The weight of the carcass after the trachea, heart, liver, gizzard, muscular stomach, lungs, abdominal fat, esophagus, crop, intestines, spleen, pancreas, gallbladder, reproductive organs, and the contents of the stomach and intestines were removed. All phenotypic units were in grams.

Measurement of Biochemical Indices and Fatty Acid in Pectoral Muscle

The MA supplementation group and the control group each selected 15 pectoral muscle samples. The TG (Kit ID: A110-1-1) and TCHO (Kit ID: A111-1-1) contents of these samples were measured using assay kits (Nanjing Jiancheng Bioengineering Institute Co., Ltd., Nanjing, China), and the PLIP (Kit ID: PP7200) contents were determined with commercially available kits (Beijing Deliman Biochemical Technology Co. Ltd., Beijing, China). Approximately 0.15 grams of each chicken pectoral muscle sample was homogenized using anhydrous ethanol as the homogenization medium. The supernatant was collected for analysis using experimental procedures previously reported in detail (Liu et al., 2019). The FA composition of freeze-dried chicken pectoral muscle powder was determined by gas chromatography, on an Agilent 7890A gas chromatography system (Agilent Technologies Inc., Santa Clara, CA) using methyl undecanoate as internal standard. The peak area normalization method was used to calculate the content (percentage) of each FA (Cui et al., 2022).

Transcriptomic Analysis

The MA supplementation group and the control group each collected 15 pectoral muscle samples. Total RNA was extracted from these samples using TRIzol reagent (Invitrogen, Carlsbad, CA). The concentration and integrity of the extracted total RNA samples were assessed by measuring the absorbance ratio at A260/A280 by spectrophotometry on a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) and the quality was further assessed after by agarose gel (1%) electrophoresis. RNA-sequencing was performed on an Illumina NovaSeq 6000 S2 (Illumina, San Diego, CA) by Suzhou Bionovegene Co., Ltd., Jiangsu China.

The R (version 4.3.2) package "DEseq2" (McDermaid et al., 2019) was used to identify DEGs between the control group and MA supplementation group. Genes with a $|\log2(\text{fold change})| \geq 0.585$ and $P < 0.05$ were considered differentially expressed (Xia et al., 2020). Hierarchical clustering analysis was performed to determine the variability and repeatability of the samples and a volcano plot was used to visualize the overall distribution of DEGs.

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Wang et al., 2017) in National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Chicken: CRA016343) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa-chicken.

Weighted Gene Co-expression Network Analysis

The "WGCNA" package (Langfelder and Horvath, 2008) in R (version 4.3.2) was used to identify co-expression modules and hub genes associated with the MA and TG phenotypes. Before performing WGCNA, the genes obtained by transcriptome sequencing were filtered to remove genes whose average expression level was less than 1. To construct a weighted gene co-expression network, we calculated the pairwise correlation coefficients between all pairs of genes across the samples. The correlation matrix was then transformed into an adjacency

matrix using a soft-thresholding power function. The appropriate soft-thresholding power was selected based on the scale-free topology criterion. Subsequently, the adjacency matrix was used to define the topological overlap matrix (**TOM**), which assesses the interconnectedness between genes. Hierarchical clustering was performed using the TOM to identify co-expression modules. Module eigengenes, representing the first principal component of each module, were calculated to summarize the gene expression patterns within each module. Finally, we assessed the correlation between module eigengenes and the phenotype of interest to identify modules significantly associated with the phenotype.

Correlation Analysis

The overlapping co-expressed genes identified at the intersection between DEGs and WGCNA-derived hub genes associated with MA and TG content were further evaluated using tools from OmicShare (https://www.omicshare.com/tools) and those with an absolute correlation coefficient greater than 0.4 with MA and TG ($P < 0.05$) were retained (Jin et al., 2022).

Functional Enrichment Analysis

The identified DEGs and WGCNA-derived hub genes were subjected to Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathway enrichment analysis using the OmicShare tools (www.omicshare.com/tools). KEGG pathways categories with a $P \leq 0.05$ were considered significantly enriched.

Statistical Analysis

The data (Slaughter performance, lipid metabolism, fatty acid composition, expression levels of hub genes) obtained in this study are expressed as the mean ± standard deviation (**SD**). Tukey's test was performed for multiple comparisons of means at a significance level of $P < 0.05$ using the GraphPad Prism 9.5 software (GraphPad Software Inc., San Diego, CA).

RESULTS

Slaughter Performance and Lipid Metabolism Analysis

The differences in body weight, carcass weight, eviscerated weight, and pectoral muscle weight of chickens fed the basal diet or MA-supplemented diet were negligible ($P > 0.05$; Figure 1A).

To investigate lipid metabolism in pectoralis muscle tissue of chickens from the control and supplementation groups, we measured the relative content of TGs, PLIPs, and TCHO in the pectoralis muscle tissue samples. The results revealed significant differences in TG content between the control and supplementation groups, as shown in Figure 1B. The TG content in the supplementation group was significantly higher ($P < 0.01$) than that in the control group. However, there were no significant differences ($P > 0.05$) in the relative PLIP and TCHO contents between the two groups.

Analysis of the Fatty Acid Composition of the Pectoral Muscle

The differences in the relative contents of FAs between the two groups were further determined, including a total of 27 detected FAs. Among them, 22 FAs exhibited significant differences between the two groups (Table 2). The content of MA in the supplementation group showed a significant difference compared to the control group, with higher levels detected in the supplementation group. The results in Figure 2 show that the levels of palmitic acid, an end product of FA *de novo* synthesis, as well as the two n6 FAs, linoleic acid (**LA**) and arachidonic acid (**AA**), were significantly higher in the supplementation group compared to the control group ($P < 0.01$). On the other hand, only the levels of linolenic acid (**ALA**), an n3 FA, showed a significant difference ($P < 0.01$), while the contents of the n3 FAs docosahexaenoic acid (**DHA**) and eicosapentaenoic acid (**EPA**) were not significantly different between the two groups ($P > 0.05$). Moreover, the contents of SFAs, MUFAs, and PUFAs were significantly higher in the supplementation group compared to the control group

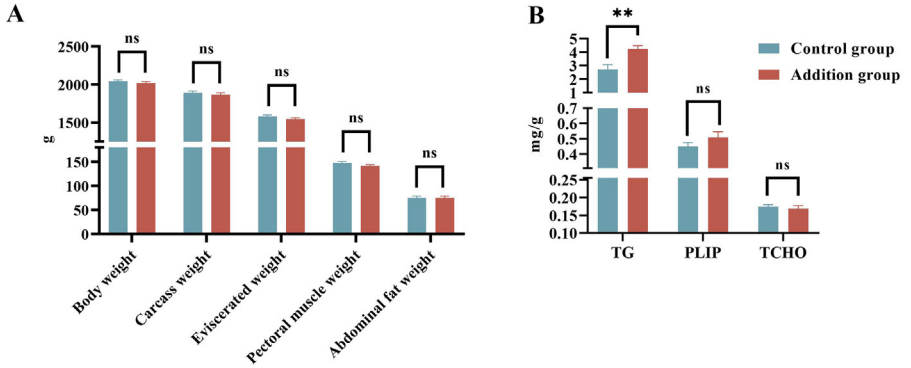

Figure 1. (A) Comparison of the mean (±SD) values for Slaughter performance between the supplementation group and control group of broiler chickens. (B) Comparison of the mean (±SD) values for Lipid metabolism between the supplementation group and control group of broiler chickens.

Table 2. Phenotypes of Jinling hua chickens in different groups (mg/g).

Phenotypes	Control	MA	*P*-value
C8:0	0.008 ± 0.001	0.008 ± 0.001	0.2
C10:0	0.018 ± 0.002	0.020 ± 0.003	0.13
C12:0	0.011 ± 0.002	0.014 ± 0.002	2E-04
C14:0	0.098 ± 0.032	0.146 ± 0.030	2E-04
C14:1	0.018 ± 0.008	0.028 ± 0.006	7E-04
C15:0	0.016 ± 0.005	0.022 ± 0.003	2E-04
C16:0	6.945 ± 2.029	8.948 ± 1.411	4.4E-03
C16:1	0.823 ± 0.300	1.064 ± 0.206	0.02
C17:0	0.030 ± 0.009	0.041 ± 0.007	8E-04
C18:0	3.270 ± 0.913	4.218 ± 0.526	1.8E-03
C18:1n9c	8.113 ± 2.756	10.921 ± 2.031	4.1E-03
C18:2n6c	5.683 ± 1.677	7.499 ± 1.207	2.3E-03
C18:3n3	0.283 ± 0.100	0.376 ± 0.068	6.7E-03
C20:0	0.047 ± 0.014	0.065 ± 0.015	2.2E-03
C20:1	0.086 ± 0.028	0.120 ± 0.019	8E-04
C21:0	0.256 ± 0.070	0.336 ± 0.051	1.4E-03
C20:3n6	0.369 ±0.108	0.492 ± 0.050	4E-04
C20:4n6	1.763 ± 0.555	2.225 ± 0.289	8.4E-03
C20:3n3	0.024 ± 0.008	0.034 ± 0.006	6E-04
C22:0	0.046 ± 0.012	0.058 ± 0.009	5.5E-03
C20:5n3	0.122 ± 0.042	0.139 ± 0.023	0.17
C22:1n9	0.024 ± 0.008	0.030 ± 0.005	0.02
C22:2	0.013 ± 0.004	0.019 ± 0.003	3E-05
C23:0	0.038 ± 0.013	0.050 ± 0.010	0.01
C24:0	0.059 ± 0.018	0.052 ± 0.014	0.25
C24:1	0.095 ± 0.023	0.112 ± 0.017	0.02
C22:6n3	0.224 ± 0.069	0.271 ± 0.057	0.06
SFA	10.840 ± 3.071	13.977 ± 1.979	2.8E-03
MUFA	9.159 ± 3.094	12.275 ± 2.252	4.3E-03
PUFA	8.480 ± 2.351	11.056 ± 1.446	1.3E-03
PUFA/SFA	0.784 ± 0.031	0.793 ± 0.042	0.51
n-3 PUFA	0.653 ± 0.185	0.820 ± 0.095	4.6E-03
n-6 PUFA	7.814 ± 2.169	10.217 ± 1.362	1.3E-03
n-6/n-3	12.040 ± 0.910	12.453 ± 0.808	0.21

Values are expressed as means ± standard deviation, $n = 15$ for control group analysis, $n = 14$ for MA group analysis. C8:0, Caprylic acid; C10:0, Capric acid; C12:0, Lauric acid; C14:0, Myristic acid; C14:1, Myristoleic acid; C15:0, Pentadecanoic acid; C16:0, Palmitic acid; C16:1, Palmitoleic acid; C17:0, Heptadecanoic acid; C18:0, Stearic acid; C18:1n9c, Oleic acid; C18:2n6c, Linoleic acid; C18:3n3, Linolenic acid; C20:0, Arachidic acid; C20:1, cis-11-Eicosenoic acid; C21:0, Henicosanoic acid; C20:3n6, cis-8,11,14-Eicosatrienoic acid; C20:4n6, Arachidonic acid; C20:3n3, cis-11,14,17-Eicosatrienoic acid; C22:0, Behenic acid; C20:5n3, cis-5,8,11,14,17-Eicosapentaenoic acid; C22:1n9, Erucic acid; C22:2, cis-13,16-Docosadienoic acid; C23:0, Tricosanoic acid; C24:0, Lignoceric acid; C24:1, Nervonic acid; C22:6n3, cis-4,7,10,13,16,19-Docosahexaenoic; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; n-3 PUFA, omega-3 PUFA; n-6 PUFA, omega-6 PUFA.

($P < 0.01$), but there was no significant difference in the ratios of PUFA/SFA and n6/n3 between the two groups ($P > 0.05$).

The MA content was positively correlated with the contents of palmitic acid, LA, ALA, n3, n6, SFAs, and UFAs (Figure 3), while the contents of AA, DHA and EPA were moderately correlated with other FAs.

KEGG Enrichment Analysis of DEGs

The transcriptome sequencing data of 15 samples of the supplementation group and 14 samples of the control group (Table S1) were subjected to differential gene expression analysis to identify DEGs. The volcano plot in Figure 4A shows the identified DEGs between the supplementation and control groups. Compared to the control group, a total of 147 and 2,003 genes were found to be upregulated and downregulated, respectively, in the supplementation group (Figure 4B). We also examined several pathways in which different genes were enriched, mainly including lipid metabolism, signaling molecules and interaction, and immune system (Figure 4C). Lipid metabolic pathways include the Steroid biosynthesis pathway, which contains *MSMO1, SQLE, LBR, DHCR24, SOAT1, NSDHL, LIPA,* and ENSGALG00000023824, all of which were downregulated. We also detected significant enrichment of biological processes related to immune system in DEGs in muscle. These included the Toll-like receptor signaling pathway (P = 5.08E-08), Leukocyte transendothelial migration (P = 1.82E-07), Chemokine signaling pathway (P = 3.1E-06), T cell receptor signaling pathway (P = 5.06E-5), and NOD-like receptor signaling pathway (P = 0.00013) for DEGs in muscle (Table S2). Overall, these results suggest that dietary supplementation with MA is not only related to lipid metabolism in the body, but also that there are potential connections between the immune system and disease. Signaling pathways associated with cell connections included Cytokine-cytokine receptor interaction (P = 9.57E-17), Regulation of actin cytoskeleton (P = 4.23E-05), Cell adhesion molecules (P = 5.74E-05).

Weighted Gene Co-Expression Network Construction and Module Detection

A weighted gene co-expression network was constructed to identify key genes associated with MA regulation, which resulted in the identification of 11,867 genes. Based on the clustering analysis of the samples, no abnormal samples were identified (Figure 5A). After determining a soft threshold at R2 > 0.85 (Figure 5B), 8 co-expression gene modules were identified using the dynamic tree cut method (Figure 5C).

To identify co-expression modules associated with traits of MA, we evaluated the relationship of MA to module eigengene (ME). MA was significantly and positively correlated with the green module (r = 0.55, P = 0.002), magenta module (r = 0.42, P = 0.02) and tan module (r = 0.5, P = 0.006) (Figure 5D). Additionally, we found that TG was significantly positively correlated with the magenta module (r = 0.46, P = 0.01) and significantly negatively correlated with the black module (r = −0.46, P = 0.01) and blue module (r = −0.49, P = 0.007).

Considering that there were only three MA-related module genes, we combined the three module genes for pathway enrichment analysis, and the results showed that Ubiquitin mediated proteolysis (P = 0.007), mTOR signaling pathway (P = 0.009), Insulin signaling pathway (P = 0.029), and NOD-like receptor signaling pathway (P = 0.041) were enriched(Figure 6A).

The three TG-related module genes were enriched in multiple signaling pathways (Figure 6B, Table S3), and the classical lipid metabolism pathways Fatty acid

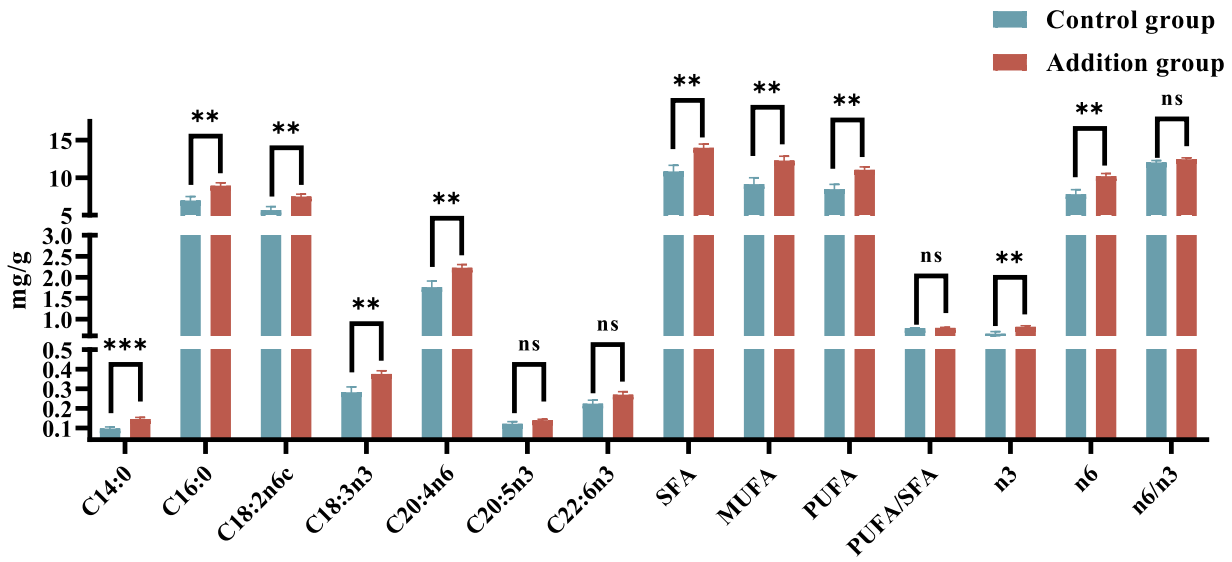

Figure 2. Comparison of the mean (±SD) values for fatty acids between the supplementation group and control group of broiler chickens. C14:0, Myristic acid (MA); C16:0, Palmitic acid; C18:2n6c, Linoleic acid (LA); C18:3n3, Linolenic acid (ALA); C20:4n6, Arachidonic acid (AA); C20:5n3, eicosapentaenoic acid (EPA); C22:6n3, docosahexaenoic acid (DHA); SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; n-3, omega-3; n-6, omega-6.

biosynthesis ($P = 0.002$) and Cholesterol metabolism ($P = 0.021$) were also enriched. After pathway classification, it was found that the Endocrine system includes Insulin signaling pathway ($P = 1.95E\text{-}06$), Adipocytokine signaling pathway ($P = 3.2E\text{-}05$), Glucagon signaling pathway ($P = 0.001$). Signal transduction includes FoxO signaling pathway ($P = 6.84E\text{-}05$), ErbB signaling pathway ($P = 0.0001$), AMPK signaling pathway ($P = 0.001$), mTOR signaling pathway ($P = 0.004$), and TGF-beta signaling pathway ($P = 0.044$).

Identification of Genes Associated with MA and TG

The five overlapping genes at the intersection of DEGs and WGCNA-derived hub genes associated with the MA content were identified as Basic Helix-Loop-Helix Family Member E40 (***BHLHE40***), Major Facilitator Superfamily Domain-Containing Protein 1 (***MSL1***), PLAG1 like zinc finger 1 (***PLAGL1***), Serine/Arginine-Rich Splicing Factor 4 (***SRSF4***), and

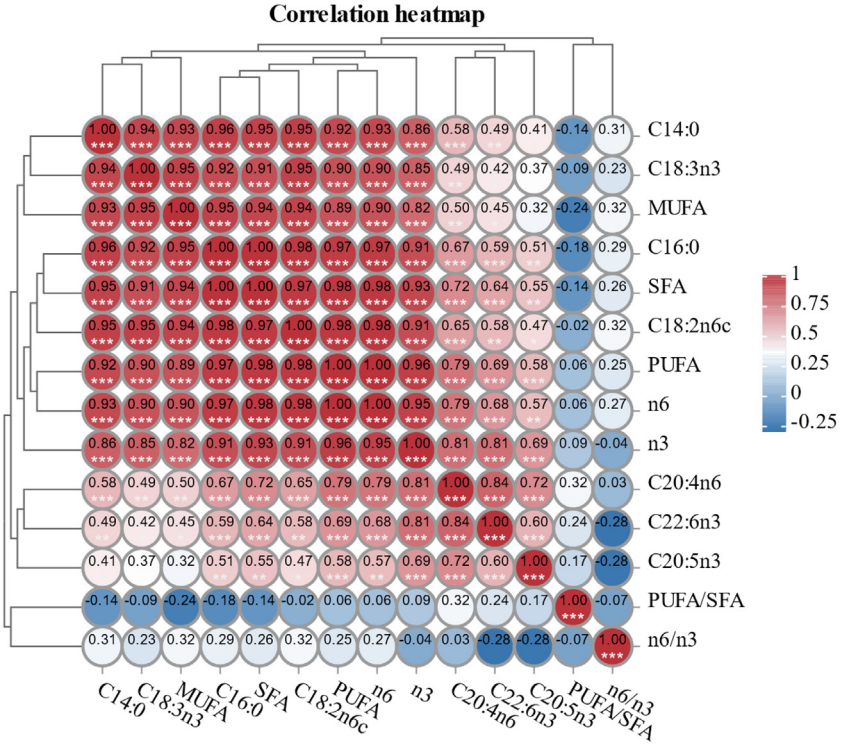

Figure 3. Correlation analysis of key fatty acids. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, blue indicates positive correlation, red indicates negative correlation.

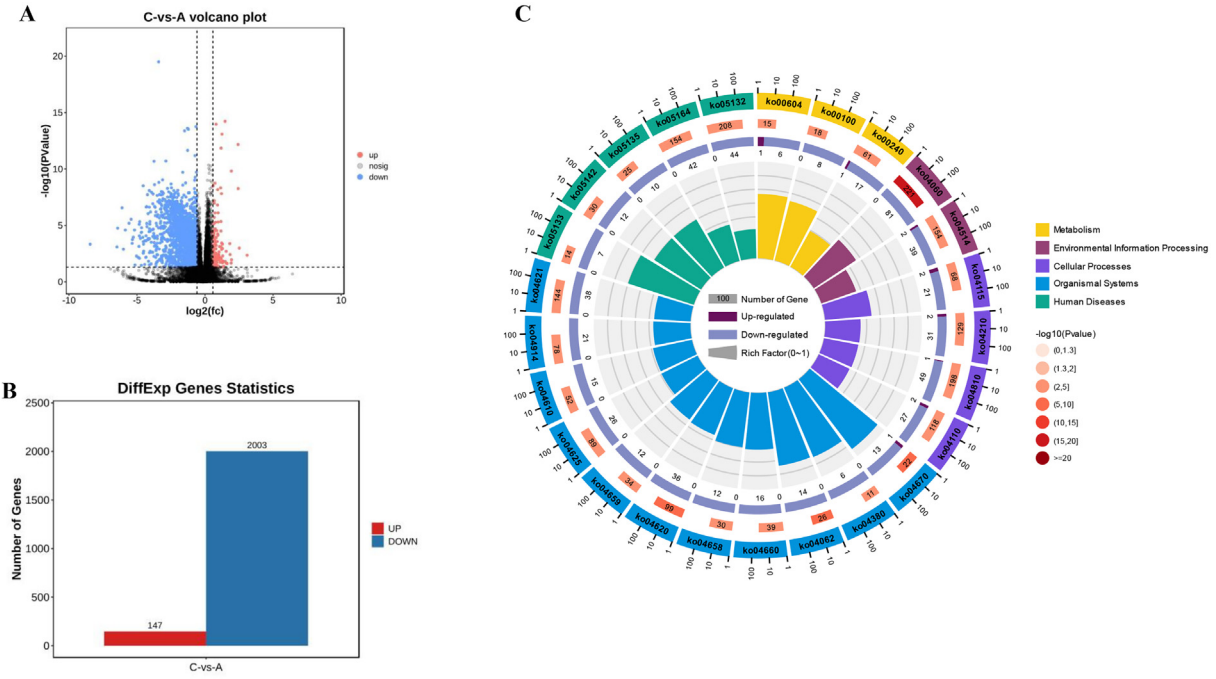

Figure 4. (A) Volcano plot for control group vs. supplementation group DEGs. (B) Histogram of DEGs in the control group and supplementation group. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis of DEGs in the control group vs. supplementation group. DEGs: differentially expressed genes.

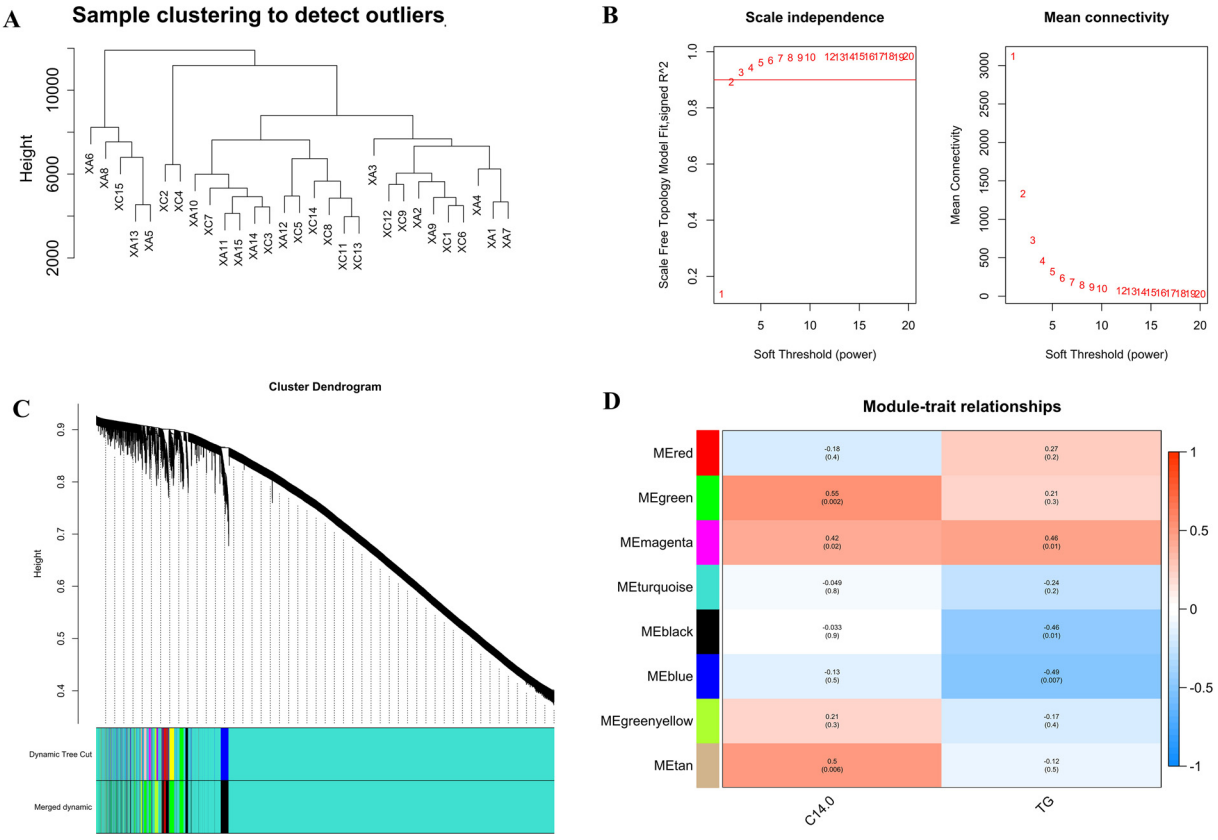

Figure 5. Co-expression network analysis of genes associated with MA and TG contents in pectoral muscle in the supplementation group and control group of broiler chickens. (A) Sample clustering. (B) Scale independence and mean connectivity. (C) The cluster of genes. (D) Module heatmap, the gene modules and trait relationships (red indicates positive correlation; blue indicates negative correlation).

ENSGALG00000026875 (Figure 7A). Further analysis revealed a significant correlations ($0.4 < cor < 0.8$) between these five genes and MA content. Specifically, *BHLHE40* (cor = 0.653), *MSL1* (cor = 0.581), *SRSF4* (cor = 0.421) and ENSGALG00000026875 (cor = 0.538) were found to be positively correlated, while only *PLAGL1* (cor = -0.462) was negatively correlated with MA content. Similarly, a total of 28 genes were found to

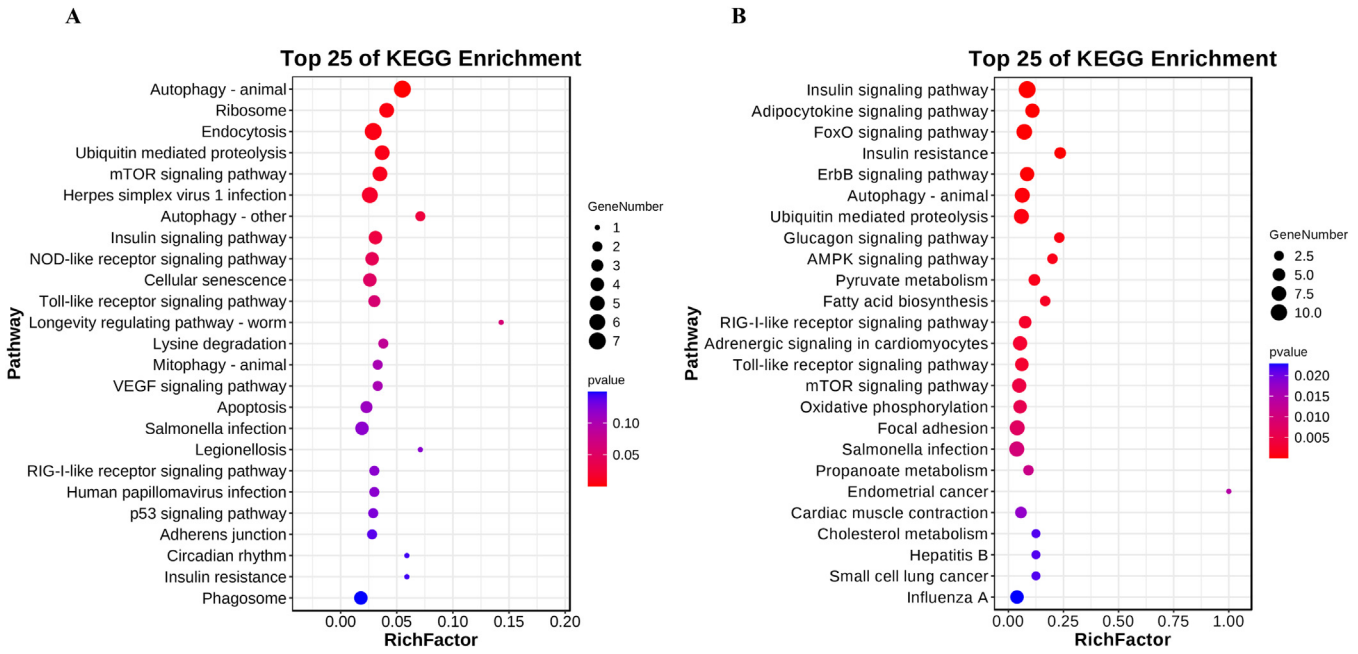

Figure 6. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of genes associated with MA and TG contents in the WGCNA results. (A) KEGG pathway enrichment analysis of overlapping genes associated with MA content ($P < 0.05$). (B) KEGG pathway enrichment analysis of overlapping genes associated with TG content ($P < 0.05$).

be correlated with TG content as shown in detail in Table 3, where 26 out of the 28 genes showed significant correlations. Notably, *PLAGL1* emerged as both a DEG and a WGCNA-derived hub gene for both MA and TG contents, exhibiting significant negative correlations with both variables. These findings highlight the crucial role played by this gene in regulating TG content during MA production. By referring to relevant literature, we screened the genes closely related to lipid metabolism, Choline Kinase Alpha (***CHKA***), Krüppel-Like Factor 5 (***KLF5***), TGFB induced factor homeobox 1 (***TGIF1***), among the 28 genes related to TG according to gene function. The expression of these three genes and five MA-related hub genes in the control group and the addition group were further observed (Figure 7B).

DISCUSSION

IMF is closely related to the tenderness, flavor and nutritional value of meat, and FAs are important components of IMF (Cao et al., 2023). The influence of FA content and muscle composition on chicken quality has become one of the hot topics in poultry breeding and meat science research. Therefore, research on the regulatory mechanism of the production of FAs can provide a theoretical basis for improving the quality of chicken products and has important practical implications for improving the quality and efficiency of broiler meat production and meeting the current demands of consumers.

PUFAs are oxidized to form volatile compounds, such as aldehydes, which give meat its unique flavor and

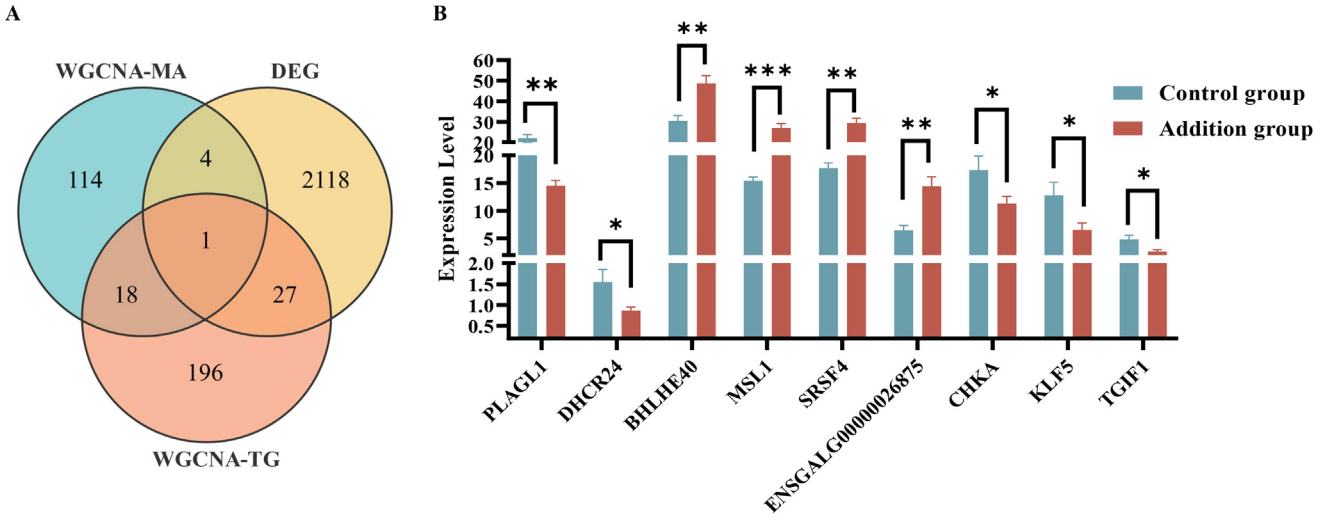

Figure 7. Venn diagram of DEGs and genes associated with MA and TG contents in the WGCNA results. (A) Venn diagram of DEGs and genes associated with MA and TG contents in the WGCNA results. (B) Expression levels of hub genes in the supplementation group and control group.

Table 3. The correlation coefficients between TG, MA, and the DEG.

Phenotypes	ID	Name	Cor
MA	ENSGALG00000008308	*BHLHE40*	0.653
	ENSGALG00000026875	-	0.538
	ENSGALG00000030739	*MSL1*	0.581
	ENSGALG00000013754	*PLAGL1*	-0.462
	ENSGALG00000050314	*SRSF4*	0.421
	ENSGALG00000004144	-	-0.617
	ENSGALG00000001951	-	-0.439
	ENSGALG00000040957	*AGO2*	-0.501
	ENSGALG00000039575	*AMD1*	-0.482
	ENSGALG00000007254	*ARHGAP12*	-0.518
	ENSGALG00000011236	*BEGAIN*	0.542
	ENSGALG00000036754	*CHKA*	-0.412
	ENSGALG00000038265	*CREB5*	-0.442
	ENSGALG00000032257	*CSNK1E*	-0.6
	ENSGALG00000016231	*DDX3X*	-0.59
	ENSGALG00000015181	*GAREM1*	-0.43
	ENSGALG00000007383	*HSPB8*	-0.552
	ENSGALG00000016927	*KLF5*	-0.592
	ENSGALG00000006260	*KLHL30*	-0.446
	ENSGALG00000013886	*LDLRAD4*	-0.503
	ENSGALG00000012277	*MAFF*	-0.507
TG	ENSGALG00000015681	*NRIP1*	-
	ENSGALG00000004613	*P2RX5*	-0.459
	ENSGALG00000052355	*PDE12*	-0.63
	ENSGALG00000013754	*PLAGL1*	-0.471
	ENSGALG00000020742	*PPRC1*	-0.642
	ENSGALG00000017244	*PRSS23*	-
	ENSGALG00000013209	*SLC39A6*	-0.545
	ENSGALG00000017053	*SPART*	-0.717
	ENSGALG00000014795	*TGIF1*	-0.567
	ENSGALG00000007324	*TP63*	-0.516
	ENSGALG00000011389	*TRAF3*	-0.445
	ENSGALG00000020067	*VASH2*	-0.53

Genes associated with MA: *BHLHE40*: Basic Helix-Loop-Helix Family Member E40; *MSL1*: Major Facilitator Superfamily Domain-Containing Protein 1; *PLAGL1*: Pleiomorphic Adenoma Gene-Like 1; *SRSF4*: Serine/Arginine-Rich Splicing Factor 4.

Genes associated with TG: *AGO2*: Argonaute 2, RISC catalytic component; *AMD1*: Adenosylmethionine decarboxylase 1; *ARHGAP12*: Rho GTPase activating protein 12; *BEGAIN*: Brain enriched guanylate kinase associated; *CHKA*: Choline kinase alpha; *CREB5*: cAMP responsive element binding protein 5; *CSNK1E*: Casein kinase 1 epsilon; *DDX3X*: DEAD-box helicase 3X; *GAREM1*: GRB2 associated regulator of MAPK1 subtype 1; *HSPB8*: Heat shock protein family B (small) member 8; *KLF5*: Kruppel like factor 5; *KLHL30*: Kelch like family member 30; *LDLRAD4*: Low density lipoprotein receptor class A domain containing 4; *MAFF*: MAF bZIP transcription factor F; *NRIP1*: Nuclear receptor interacting protein 1; *P2RX5*: Purinergic receptor P2×5; *PDE12*: Phosphodiesterase 12; *PLAGL1*: PLAG1 like zinc finger 1; *PPRC1*: Peroxisome proliferator-activated receptor gamma, coactivator-related 1; *PRSS23*: Protease, serine 23; *SLC39A6*: Solute carrier family 39 member 6; *SPART*: Spastic paraplegia 20 (Troyer syndrome); *TGIF1*: TGFB induced factor homeobox 1; *TP63*: tumor protein p63; *TRAF3*: TNF receptor associated factor 3; *VASH2*: Vasohibin 2.

aroma characteristics (Miranda et al., 2021; Dyall et al., 2022). In addition, PUFAs play a regulatory role in animal lipid metabolism and are key bioactive compounds affecting lipid metabolism in animal tissues. MUFAs can reduce cholesterol, TGs and low-density lipoprotein cholesterol (**LDL-C**) in human blood, help prevent cardiovascular diseases like atherosclerosis, and reduce the risk of cancer to some extent (Cao et al., 2022).

MA is a SFA commonly found in coconut oil, palm oil and animal fats. As a FA, MA plays a variety of roles in livestock and poultry. First, after digestion and absorption, it can be metabolized to ATP to provide energy (Heuel et al., 2021). Second, as a raw material for the synthesis and storage of body fat, it participates in the synthesis of FAs and is stored as fats in the form of TGs to serve as an energy source (Zhou et al., 2023). Third, it affects the fluidity and permeability of the cell membrane to regulate the exchange and transfer of substances inside and outside the cell. In contrast to the above-mentioned extensive studies on UFAs, there are few studies on SFAs, and even fewer on nutrient supplementation. This is likely due to the excessive intake of SFAs that may cause an imbalance in the proportion of FAs, resulting in abnormal lipid synthesis, ultimately affecting lipid content and tissue structure, thus breaking the nutritional balance of livestock and poultry. Furthermore, excessive intake of SFAs may lead to insulin resistance, fatty liver and other metabolic conditions, which have a negative impact on the growth, development and immune function of livestock and poultry (Funaki, 2009).

However, the results of this study show that at the appropriate dosage and ratio, under the condition of ensuring nutritional balance and comprehensiveness of feed nutrients, the supplementation with 0.04% MA did not adversely affect the ratio of FAs PUFA/SFA (n6/n3). But led to a significant increase in the TG content. Relevant studies have shown that with the reduction of the TG content, the total amount of MA, as a constituent of TGs, also decreased significantly, and its rate of decrease was the greatest among all the FA components (Taya et al., 2023). Previous studies have confirmed that TGs stored in adipose cells are the main components of IMF, and increased TG content indicates increased IMF content, thus an appropriate increase of IMF content can increase the taste and tenderness of muscles (Cui, 2022). The content of PUFAs, such as LA, ALA, and AA, may increase the content of E-2-nonenal, 2, 4-decadienal, and other volatile flavor compounds, which have a favorable effect on the flavor of meat (Díaz et al., 2005).

Considering that the supplementation of MA not only changed the content of MA, but also significantly increased the content of TGs, in this study transcriptome sequencing was performed on samples from the supplementation and control groups to identify the genes and pathways involved. The gene 24-Dehydrocholesterol Reductase (*DHCR24*) encodes a protein involved in the steroid biosynthesis pathway, and changes in its expression have been demonstrated in several abdominal fat studies to cause changes in fat deposition (Mu et al., 2019; Wei et al., 2024). DEGs are also enriched in multiple immune signaling pathways mediated by Toll-like receptors (TLRS), which have been identified as dominant innate immune receptors that play a key role in immune system development and function (Aluri et al., 2021; Shafeghat et al., 2022). It has been suggested that nutrient supplementation may play an important role in the regulation of the immune system. In the pathway analysis of WGCNA modules, both MA and TGs influenced the mTOR signaling pathway, which promotes lipid synthesis by activating *SREBP* (Chu et al., 2022). *SREBP* encodes a transcription factor

that primarily regulates the transcription of genes associated with cholesterol and lipid synthesis, such as acetyl-CoA carboxylase (***ACC***) and fatty acid synthase (***FASN***). All-trans retinoic acid (**ATRA**) was found to regulate the mTOR signaling pathway, which is involved in lipid metabolism, by inhibiting the expression of *AKT2* and the adipogenic transcription factors SREBP1, ACC, and FAS (Zhang et al., 2022). Three enzymes that play a key role in the lipid synthesis pathway and participate in the synthesis of FAs. Following their activation, the mTOR complex 1 (**mTORC1**) and mTOR complex 2 (**mTORC2**) facilitate the accumulation of TGs by promoting adipogenesis and lipogenesis and by shutting down catabolic processes such as lipolysis and β-oxidation (Caron et al., 2015).

A total of 5 overlapping co-expressed genes identified by differential gene expression analysis and WGCNA of genes associated with MA content were selected, namely *BHLHE40, MSL1, PLAGL1, SRSF4,* and ENSGALG00000026875. *BHLHE40* encodes a transcription factor that plays an important regulatory role in FA metabolism and energy balance, which regulates the transcriptional activity of Sterol Regulatory Element-Binding Proteins (**SREBPs**), thereby regulating FA synthesis, degradation and oxidation (Tian et al., 2018). According to *in vitro* functional analyses, BHLHE40 was shown to negatively control FA oxidation in cultured myocyte (Takeshita et al., 2012). Knockdown of *BHLHE40* mRNA can increase the expression level of FA oxidation target genes (Chung et al., 2015). Both Differentiated Embryonic Chondrocyte-expressed Gene 1 (***DEC1***) and *BHLHE40* belong to the helix-loop-helix (**HLH**) transcription factor family. They play an important role in regulating the biological clock, cell cycle, cell differentiation, and other biological processes. ACC is a key enzyme in FA synthesis, responsible for the conversion of acetyl-CoA to acyl-CoA, which is one of the rate-limiting steps in FA synthesis. The expression level of *BHLHE40* is negatively correlated with the expression of *ACC*, and the overexpression of *BHLHE40* can inhibit the expression of *ACC*, thus reducing the synthesis of FAs (Shen et al., 2014).

Unlike MA, the effects of TGs were concentrated on 28 genes, including *CHKA*, which is involved in metabolic pathways and glycerophospholipid metabolism, and plays an important role in lipid metabolism and energy homeostasis (Xu et al., 2021; Xu et al., 2022). Also, among these 28 genes is *KLF5*, which binds to SREBP-1 and enhances the SREBP-1-mediated increase in the promoter activity of FASN (Lee et al., 2009). Transient transfection with mouse *TGIF1* mRNA lowered levels of cholesterol ($P < 0.001$), TGs ($P < 0.001$), and apolipoprotein B ($P < 0.05$) in the cell media by ~40%, along with the mRNA levels of some key genes involved in lipid metabolism (Härdfeldt et al., 2019). These findings are consistent with our results, *TGIF1* expression is negatively correlated with TG content. *TGIF1* interacts with LXRα and *TGIF1* null mice have increased expression of the two LXRα target genes apolipoproteins (Apo) c2 and a4. Also, *TGIF1* was recently found to function as a transcriptional repressor of the cholesterol esterifying enzyme acyl-coenzyme A:cholesterol acyltransferase 2, encoded by the gene name *SOAT2* (Pramfalk et al., 2015).

The *PLAGL1* gene encodes a zinc finger protein involved in regulating gene expression, which plays a role in various cellular processes, such as cell proliferation, differentiation, and apoptosis (Shi et al., 2022). A family-based association analysis study of high density lipoprotein cholesterol (**HDL-C**) in 907 patients revealed that SNP rs2257104 in *PLAGL1* at approximately 143 cM was associated with multivariable adjusted HDL ($P = 0.03$) (Yang et al., 2005). PLAGL1 directly binds to the PPARγ promoter to regulate lipid synthesis (Riegl et al., 2023). Diacylglyceryltransferase-1 (***DGAT1***) encodes a key enzyme that controls TG synthesis in adipose tissue. *PLAGL1* has been identified as a DEG in DGAT1-knockout mice, and it has been speculated that *PLAGL1* plays a role in TG synthesis along with *DGAT1* (Ying et al., 2017).

This study has shown that at the specific inclusion level of 0.04%, the MA supplement resulted in a proportional increase in PUFA content that led to similar PUFA/SFA ratios between the MA supplemented group and the control group. The increased PUFA content in the MA supplemented meat may potentially improve its flavor profile. However, this study has certain limitations, as only one time point and concentration were analyzed, and subsequent dynamic monitoring at multiple time points is required to fully monitor the changes of related genes and regulatory networks.

CONCLUSION

Dietary MA supplementation for 56 days had little adverse effect on the body weight, carcass weight, eviscerated weight, and pectoral muscle weight of broiler chickens, instead it enriched the meat with LA, ALA, and AA and increased the TG content. Therefore, its supplementation with MA is appropriate for health-conscious consumers. Key regulatory genes, such as *BHLHE40* and *PLAGL1*, were identified through differential gene expression analysis, WGCNA, and correlation analysis. These findings provide valuable lipid phenotype and gene expression data for the nutritional supplementation of MA in chickens. Further insights into the roles of the central regulatory factor *PLAGL1* and other potentially detectable genes could provide information for the conversion of increased MA content into TG.

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DISCLOSURES

The authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this study.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.104038.

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