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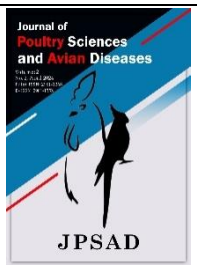

Investigating the impact of various fat supplements and their levels on the expression of lipid metabolism-regulated genes in the liver tissue of broiler chickens

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A B S T R A C T

Over the last few decades, progress in molecular biology and genetic technologies has allowed scientists to explore the mechanisms of lipid metabolism in chickens. Through techniques like RNA sequencing and gene expression profiling, researchers can now investigate how dietary fat supplements impact the expression of particular genes related to lipid metabolism regulation. Limited research exists on the comparative impacts of fat powder and fatty acid incorporation in broiler feed on the expression of lipid metabolism-regulating genes in the liver. This study aims to identify particular genes and metabolic pathways linked to reduced abdominal fat deposition in commercial broiler chickens. The study involved 500 broiler chickens from the commercial strain Ross 308, distributed in a completely randomized layout with five different treatments and five replications. The duration of the research lasted for 42 days. Initially, the control group received no fatty acid or fat powder, whereas the other groups were supplemented with either 3% or 6% fat powder or fatty acid. The experimental period was divided into two distinct phases: the starter phase (0-21 days) and the grower phase (22-41 days). Fatty acid synthase (FASN), malic enzyme 1 (ME1), stearoyl-CoA desaturase (SCD), glycerol-3-phosphate acyltransferase 3 (GPAT3), glycerol-3-phosphate acyltransferase, mitochondrial (GPAM), CD36 molecule (CD36), carnitine palmitoyltransferase 1A (CPT1A), carnitine palmitoyltransferase 2 (CPT2), acyl-CoA oxidase 1 (ACOX1), apolipoprotein B (APOB), very low-density apolipoprotein II (apoVLDLII), peroxisome proliferator-activated receptor alpha (PPARα), peroxisome proliferator-activated receptor gamma (PPARγ) genes selected as indicators of lipid metabolism-regulation and were studied using qPCR technique. The impact of fatty acids (FA) on the regulation of genes under investigation was found to be more pronounced. However, both types of fat increased abdominal fat, with FA demonstrating a stronger effect. While the fat level was deemed significant in certain instances, the current findings and methodology suggest that a 3% fat supplement may be a more cost-effective and economical option for farm use. To further enhance the initial findings and gain a more comprehensive understanding of fat metabolism in poultry, future studies should involve comparative and correlative analyses, encompass a wider range of fat sources, and consider production traits in conjunction with genes associated with fatty acids in the liver.

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1 Introduction

In the domain of industrial poultry farming, a variety of fat sources are commonly used as feed ingredients. One traditional approach to enhancing the consistency and palatability of meal diets for broiler chickens involves incorporating small amounts of fat and oil (1). However, the response of poultry to different types of fats, whether derived from animals or plants, can vary due to fluctuations in the amount of energy that can be metabolized, which is likely influenced by the type and composition of fatty acids (2). Researchers have documented the components of oil produced for poultry feed, which may contain 75 to 95% free fatty acids along with varying levels of triglycerides. The remaining components typically comprise saturated fatty acids, phospholipids, sterols, tocopherols, carotenoids, pigments, and other fat-soluble compounds (3).

Broiler chickens often develop obesity due to their breeding for increased body weight, resulting in fat accumulation in the abdominal and visceral regions during their growth (4). Abdominal adiposity strongly correlates with overall carcass lipid content and is the primary indicator for evaluating surplus fat accumulation in broiler chickens (5). Chickens that grow rapidly exhibit a higher and faster fat accumulation rate than their slow-growing counterparts. While excessive adipose tissue reduces feed efficiency and lean meat yield in broiler chickens, it is crucial for meat flavor, as intramuscular fat enhances meat's eating quality and flavor.

The composition of dietary nutrients, such as protein and fat, can reduce fat deposition to some extent by influencing the expression of genes related to lipid metabolism (6). However, genetic engineering may be the most effective approach when it comes to decreasing fat deposition in chickens, especially considering that the heritability of abdominal fat percentage is relatively high at around 0.7 (7, 8). Nevertheless, selecting a genetically modified chicken line that combines rapid growth and reduced fat deposition is challenging due to the positive genetic correlation between these two traits (7, 9).

Calcium soaps of fatty acids, also known as fat powder, exhibit insolubility in neutral environments. The production of these substances necessitates the utilization of pH soap paste derived from the residue of vegetable oil refining plants. The inclusion of fat powder in the diets of livestock and poultry poses several challenges, including handling, storage, susceptibility to oxidation, and transportation. Mala et al. (2004) conducted a study that demonstrated a significant decline in the performance of broiler chickens when vegetable oils were substituted with calcium soaps of fatty acids (10). Tebaidian and Sadeghi (2006) introduced 5% levels of calcium soaps of fatty acids into the diets of broiler chickens and observed a notable 7% reduction in weight among chickens fed with 5% fat powder (11). They proposed that this decrease could be due to the inferior quality of the product or its reduced digestibility resulting from incomplete hydrolysis in the chickens' digestive system.

Lipid metabolism comprises intricate pathways that are governed by multiple enzymes. These enzymes are influenced by different genes, with specific genes assuming a crucial role in overseeing lipid metabolism, thereby affecting either heightened lipogenesis or lipid oxidation (12-14). Birds rely on their liver as the primary organ for lipid metabolism. The hepatocytes carry out lipid metabolism through three distinct processes. Firstly, they acquire lipids and fatty acids from the surrounding environment. Secondly, hepatocytes engage in lipogenesis, which involves the synthesis of triglycerides and fatty acids from scratch. These newly formed lipids are combined into simple lipids and stored as lipid droplets. Lastly, hepatocytes consume lipids through lipolysis, β-oxidation, and the secretion of lipids as low-density lipoproteins. This intricate process of lipid metabolism in birds is crucial for their overall energy balance and physiological functions (13). These enzymes are influenced by different genes, with certain genes assuming a crucial role in overseeing lipid metabolism, thereby affecting either heightened lipogenesis or lipid oxidation (12-14). Numerous research studies, such as those conducted by Bourneuf et al. (2006), D'Andre et al. (2013), and Resnyk et al. (2015), have been undertaken to evaluate the expression of lipid-related genes in lean and fat chicken breeds (12, 14, 15). These inquiries have revealed that a multitude of genes are implicated in lipid-related processes, and changes in their expression levels could be associated with the differences in fat deposition or leanness observed among different chicken breeds.

Chickens serve as a valuable model organism for studying abdominal obesity (16, 17), prompting the research to investigate the factors contributing to differences in abdominal fat levels among genetically similar broilers. The study also sought to pinpoint specific genes and metabolic pathways associated with decreased abdominal fat accumulation in these birds. Ultimately, the goal was to evaluate how fatty acids and fat powder affect various

aspects of liver lipid metabolism in broilers, analyze gene expression patterns, and compare the effects of these two fat sources.

2 Materials and Methods

2.1 Chickens and management

The study involved 500 broiler chickens of the commercial strain Ross 308, arranged in a fully randomized design with five treatments and five replications. Each replication consisted of 20 chickens, and the research spanned a period of 42 days. The control treatment initially did not include any fatty acid or fat powder, while the other treatments included 3% and 6% fat powder or fatty acid. The experimental period was split into the starter phase (0-21 days) and the grower phase (22-41 days). Prior to the study, the crude protein content of the feed ingredients was analyzed in the laboratory using AOAC (1984) methods to ensure proper protein levels (18). The experimental diets were formulated according to the guidelines from the NRC (1994), as detailed in Table 2 (19). Commercial poultry diets typically include an addition of fat ranging from 20 to 50 g/kg, which is determined by the prevailing prices of fat and cereal grains (20). However, a value of 60 g/kg has been selected to account for the potential impact of higher fat quantities. The formulation of rations was accomplished through the utilization of UFFDA software. Throughout the experiment, the mortality rate of birds in each enclosure was documented daily. Necropsies were performed on the birds that perished during the experiment to identify any potential lesions. Details of fatty acid and powder utilized in the current study are shown in Table 1.

Table 1. Compositions and calculated nutrient contents of starter, grower and finisher diets (g/100 g diet, as-fed basis)

Item	Starter (0 to 3 wk)			Grower (3 to 6 wk)		
	Control	3%fat	6%fat	Control	3%fat	6%fat
Soybean meal (47% CP)	33.05	32.77	32.49	22.15	24.44	24.16
Corn (8.3% CP)	62.12	54.65	47.17	70.24	62.48	55.01
Wheat bran (15.7% CP)	0.59	5.36	10.03	1	4.83	9.60
Fish meal (65% CP)	-	-	-	3.31	2	2
Fat powder or Fatty acid*	-	3	6	-	3	6
DL-Met	0.21	0.21	0.21	0.14	0.14	0.14
L-Lys	0.18	0.18	0.18	0.19	0.19	0.19
L-Thr	0.02	0.02	0.02	0.02	0.02	0.02
Salt	1.6	1.6	1.6	0.5	0.5	0.5
Calcium carbonate	1.42	1.42	1.42	1.20	1.20	1.20
Monocalcium phosphate (21% P)	1.02	1.02	1.02	1.28	1.28	1.28
Vitamin E	0.02	0.02	0.02	0.02	0.02	0.02
Premix[1]	0.50	0.50	0.50	0.50	0.50	0.50
Sum	100	100	100	100	100	100
Calculated nutrients (%)						
ME[2], Kcal/kg	2900	2900	2900	3000	3000	3000
CP	20.84	20.84	20.84	18.75	18.75	18.75
Digestible Lys	0.96	0.96	0.96	0.85	0.85	0.85
Digestible Met	0.44	0.44	0.44	0.35	0.35	0.35
Digestible Met + Cys	0.66	0.66	0.66	0.55	0.55	0.55
Digestible Thr	0.74	0.74	0.74	0.68	0.68	0.68
Crude fiber	2.72	3.07	3.41	2.54	2.87	3.22
Calcium	1	1	1	0.9	0.9	0.9
Available phosphorus	0.45	0.45	0.45	0.35	0.35	0.35

Sodium	0.2	0.2	0.2	0.15	0.15	0.15
Analyzed nutrient level, %						
Total lipid, %	2.71	5.20	7.51	3.10	5.41	7.82
Fatty acid profile (% of total fatty acid)						
SFA[3]	24.85	25.29	28.11	23.08	25.26	26.77
MUFA[4]	36.99	34.42	32.55	39.69	36.04	35.14
PUFA[5]	38.16	40.29	39.36	37.23	38.70	38.09
SFA[3]/MUFA[4]	0.67	0.73	0.71	0.58	0.70	0.76
n-6[6]	36.48	33.59	30.07	35.67	32.75	28.27
n-3[7]	1.68	6.70	9.29	1.56	5.95	9.82
n-6/n-3 ratio[8]	21.71	5.01	3.24	22.71	5.49	2.88

* fatty acid with the same composition have been replaced with fat powder.

[1]Premix (0.5%) included 11.04 mg of pantothenic acid; 35 mg of nicotinic acid; 1 mg of folic acid; 15 µg of biotin; 250 mg of choline chloride; 60 mg of Mn; 45 mg of Zn; 80 mg of Fe; 1.6 mg of Cu; 0.4 mg of I; 0.15 mg of Se; 15,000 IU of vitamin A; 3,000 IU of vitamin D3; 25 IU of vitamin E; 5 mg of vitamin K3; 2 mg of vitamin B1; 7 mg of vitamin B2; 4 mg of vitamin B6; 25 µg of vitamin B12. Per kilogram of diet.

[2]Metabolizable energy.

[3]Saturated fatty acids.

[4]Monounsaturated fatty acids.

[5]Polyunsaturated fatty acids.

[6]Omega-6 polyunsaturated fatty acids.

[7]Omega-3 polyunsaturated fatty acids.

[8]Omega-6 to omega-3 polyunsaturated fatty acid ratio.

2.2 Sampling

Throughout the study, a range of parameters, such as average weight gain, feed intake, and feed conversion ratio, were computed weekly, spanning the initial phase, growth stage, and the entire breeding period. Upon completion of the 42-day experimental timeframe, 125 birds (five from each experimental group) with weights closely aligned to the average weight of their respective cohorts were chosen for further evaluation. Each selected bird was subjected to exsanguination from the jugular vein to obtain blood specimens. The serum was separated by centrifugation at 3000xg for 10 minutes at four °C and stored at -20°C for future examination. After the decapitation, the remnants and livers were separated and measured. The method described by Mirosh et al. (1980) was employed to extract and measure the adipose tissues in the abdominal region. (21). The abdominal adipose tissue index, which represents the ratio of abdominal adipose tissue weight to remaining weight, was calculated as a percentage. The left hepatic lobe was excised, rapidly frozen in liquid nitrogen for 24 hours, and transferred to -70°C for further analysis. The weight of the cookable carcass and the composition of the carcass (liver and bile, thigh, and breast) were assessed in relation to the weight of the cookable carcass (22).

2.3 Laboratory Analysis

Serum cholesterol levels were evaluated using an enzymatic kit (Parsazmun Co. Ltd., Karaj, Iran).

2.4 RNA extraction, cDNA synthesis, and gene analysis

Total RNA was extracted from hepatic tissue using TRIzol solution (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The extracted RNA was then assessed for its quantity and purity using a NanoDrop (ND-100 UV-Vis; Nanodrop Technologies, Wilmington, DE, USA). To generate complementary DNA (cDNA), a high-performance cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was utilized, following the previously described method (23). The design of primers for qRT-PCR was accomplished using software programs such as Primer Express Software for Real-Time PCR version 3.0.1 (Thermo Fisher Scientific Inc.), Primer3Plus version 2.4.2 (24), or Primer-BLAST (17). The specific primers utilized in this study are listed in Table 2. qRT-PCR was conducted on an ABI 7500 fast instrument (Applied Biosystems, ABI 7500 fast, CA, USA) using a reaction volume of 10 µL and SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio Inc.). The cycle threshold (CT) of the candidate genes was adjusted based on the average CT of the

housekeeping genes (CT = CT [target gene] - CT [reference gene]). The differences (ΔΔCT) in the mRNA levels of the target genes were calculated for the different treatments. The fold changes in the mRNA levels of all the measured genes for the treatments and the control group were determined mathematically (Fold Change = 2^ - (ΔΔCT)).

Table 2. Primers used for quantitative reverse transcription PCR (qRT-PCR)

Gene symbol	Forward primer (5′-3′)	Reverse primer (5′-3′)	Accession no.
Fatty acid synthesis			
FASN	AGAGGCTTTGAAGCTCGGAC	GGTGCCTGAATACTTGGGCT	NM_205155
ME1	CCTCGAAGCCTTCATCCGTT	GCATCTTCAGGCCAGGTGTA	NM_204303
SCD	ACCTTAGGGCTCAATGCCAC	TCCCGTGGGTTGATGTTCTG	NM_204890
TG synthesis			
GPAT3	GGCGTGGCTCTCGTTGGTAT	CCACATGTAGGCCTCGGAGA	NM_001031145
GPAM	TGGATGCTCTCTTCTCAAATGC	AATTATGCGATCGTAGGAGATTCC	XM_015288965
Fatty acid oxidation			
CD36	ACTGCGCTTCTTCTCCTCTGA	TCACGGTCTTACTGGTCTGGTAAA	NM_001030731
CPT1A	CTTGCCCTGCAGCTTGCT	AGGCCTCGTATGTCAAAGAAAATT	NM_001012898
CPT2	GCCTTCCCTCTTGGCTACCT	TCTCAGCAATGCCCACGTATC	NM_001031287
ACOX1	GATTTTTTGCAGGCGGGTATT	CACACGCTGGTTCACCTGAGT	NM_001006205
TG transport			
APOB	TGCAAATGTCCAAGGTGCAG	ACGCAGAGCATTGCTGAAAC	NM_001044633
apoVLDLII	GGTGCAATACAGGGCATTGG	GTCACGACGTTCTCTGTCAATGA	M25774
Transcription factors			
PPARA	CAAACCAACCATCCTGACGAT	GGAGGTCAGCCATTTTTTGGA	NM_001001464
PPARG	CACTGCAGGAACAGAACAAAGAA	TCCACAGAGCGAAACTGACATC	NM_001001460
Housekeeping gene			
18SrRNA	TCCCCTCCCGTTACTTGGAT	GCGCTCGTCGGCATGTA	AF173612

FASN=fatty acid synthase; ME1=malic enzyme 1; SCD=stearoyl-CoA desaturase; GPAT3=glycerol-3-phosphate acyltransferase 3; GPAM=glycerol-3-phosphate acyltransferase, mitochondrial; CD36=CD36 molecule; CPT1A=carnitine palmitoyltransferase 1A; CPT2=carnitine palmitoyltransferase 2; ACOX1=acyl-CoA oxidase 1; APOB=apolipoprotein B; apoVLDLII=very low-density apolipoprotein II; PPARA=peroxisome proliferator-activated receptor alpha; PPARG=peroxisome proliferator-activated receptor gamma; 18SrRNA=18S ribosomal RNA.

2.5 Statistical analysis

The normal data distribution was confirmed using the SAS UNIVARIATE method (version 9.4, SAS Institute Inc., Cary, NC, USA). The SAS mixed model procedure was employed to evaluate the CT results, considering feeding treatments, stage of nutrition, and their interaction as fixed effects. In the model, chickens were considered as repeated subjects. The ΔΔCT and fold change results were tested using the SAS General Linear Model.

3 Results

The investigation focused on examining particular genes linked to the accumulation of abdominal fat and hepatic fat metabolism. This study explored the impact of various levels and sources of fat supplements on these genes. The findings obtained from this research have been comprehensively presented in Table 3 and Figures 1 to 6.

Table 3. Phenotypic traits of broiler chickens fed fatty acid or fat powder during both the starter and grower phases.

		Control		Fat Powder				Fatty acid			
				3		6		3		6	
Traits		Starter	Grower	Starter	Grower	Stater	Grower	Starter	Grower	Stater	Grower
Body weight (g)	Mean	713^{b}	1789^{B}	790^{ab}	1776^{B}	797^{ab}	1888^{A}	842^{a}	1803^{AB}	823^{ab}	1893^{A}
	SEM	7.13	13.63	15.47	22.15	7.88	15.52	16.84	14.80	12.59	7.07
Daily feed intake (g)	Mean	119^{a}	228	116^{a}	219	111^{b}	220	117^{a}	220	114^{ab}	221
	SEM	1.58	2.20	1.41	1.32	1.46	1.42	1.77	2.13	1.79	2.30
Feed conversion ratio[1]	Mean	1.51^{a}	1.88	1.29^{b}	1.82	1.37^{ab}	1.81	1.29^{b}	1.69	1.31^{ab}	1.71
	SEM	0.04	0.03	0.03	0.04	0.04	0.03	0.02	0.02	0.01	0.01
% Liver weight	Mean	2.23^{ab}	2.18^{B}	2.39^{ab}	2.22^{AB}	2.88^{a}	2.84^{A}	1.77^{b}	2.00^{B}	1.83^{b}	2.03^{B}
	SEM	0.08	0.11	0.09	0.15	0.21	0.04	0.07	0.08	0.09	0.13
% Subcutaneous fat weight	Mean	1.34^{b}	0.62^{C}	1.90^{a}	1.31^{B}	1.80^{a}	2.10^{A}	1.77^{a}	1.52^{AB}	1.61^{a}	2.01^{A}
	SEM	0.17	0.06	0.15	0.11	0.20	0.37	0.22	0.09	0.31	0.27
% Abdominal fat weight	Mean	2.06^{c}	2.66^{B}	2.12^{c}	2.51^{B}	2.95^{b}	2.33^{B}	3.21^{ab}	3.80^{A}	4.16^{a}	3.71^{A}
	SEM	0.17	0.13	0.21	0.39	0.22	0.49	0.44	0.49	0.38	0.24
% Pectoral muscle weight	Mean	30.30	29.64^{B}	39.58	39.59^{A}	34.27	33.92^{AB}	38.21	38.42^{A}	35.71	36.05^{A}
	SEM	5.41	4.98	3.22	5.55	4.30	3.29	2.51	5.17	3.73	4.53
% Gizzard weight	Mean	1.54	1.40	0.55	0.52	0.60	0.74	0.83	0.91	0.73	0.62
	SEM	0.22	0.15	0.26	0.24	0.15	0.25	0.19	0.12	0.27	0.28
Serum TC (mg/dL)	Mean	109.82	112.86	105.00	88.22	118.29	121.12	111.79	81.07	120.33	117.16
	SEM	2.95	9.18	12.16	8.77	10.19	15.27	11.21	6.19	13.84	5.91
Serum TG (mg/dL)	Mean	41.68^{b}	43.47^{B}	50.82^{ab}	66.81^{AB}	66.19^{a}	73.16^{A}	48.68^{b}	46.21^{B}	77.19^{a}	79.14^{A}
	SEM	7.75	4.21	9.26	11.38	6.99	5.71	12.10	3.97	7.68	14.61

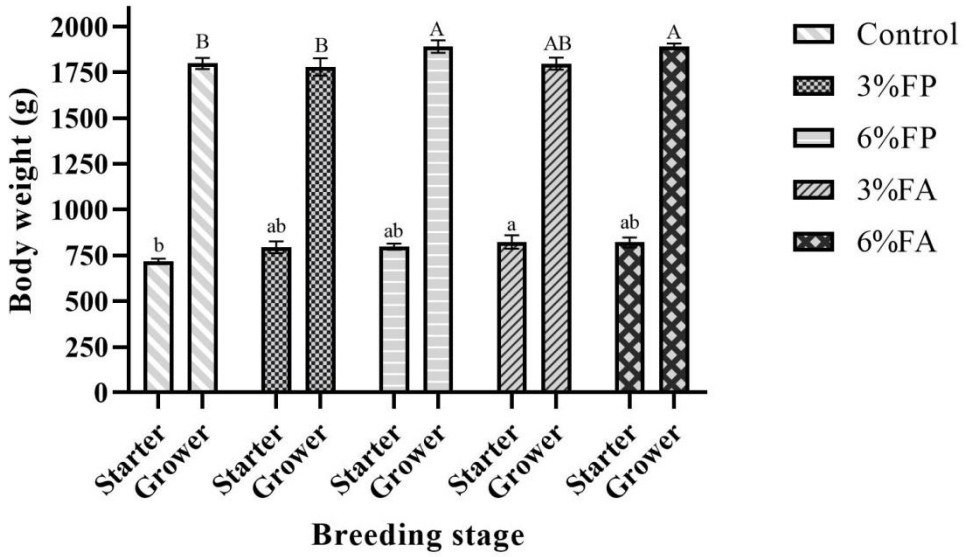


Figure 1. Body weight (g) of the broiler chickens fed experimental diets (FP= fat powder; FA= fatty acids) at two levels (3% and 6%). A–B Significantly different treatments in the grower phase are denoted by superscript letters with different subscripts at a significance level of $p<0.05$. a–b Significantly different treatments in the starter phase are denoted by superscript letters with different subscripts at the significance level of $p<0.05$. The error bars show SEM.

3.1 Phenotypic Characterization

Figure 1 illustrates the growth pattern of chickens during the starter and grower periods. Among the different groups, the chickens in the 3% fatty acid group had the highest body weight during the starter period, while the Control group had the lowest body weight. However, during the grower period, the highest body weight was observed in the 6% FA and 6% FP groups. On the other hand, the Control and 3% FP groups had the lowest body weight values, as shown in Figure 1 and Table 3.

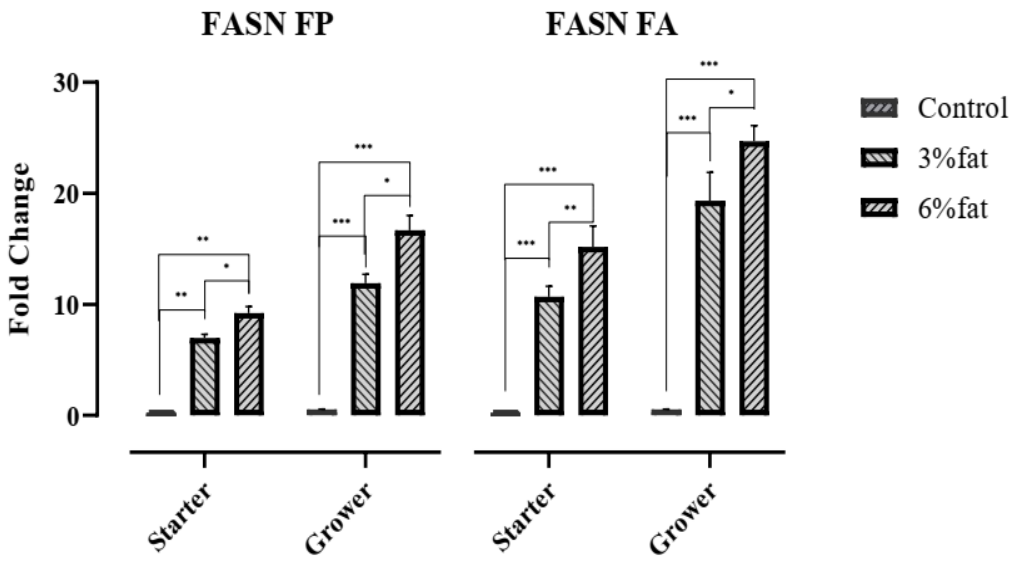
Interestingly, despite consistent daily feed intake across all groups during the grower phase, the chickens in the 6%FP group exhibited a lower feed consumption during the starter phase, as indicated in Table 3. Upon analyzing the data presented in Table 3, it becomes apparent that there were no significant variations in the percentage of liver weight among the different experimental groups, except for the 6 %FP group, which displayed higher values in both periods.

Furthermore, the control group demonstrated the lowest proportion of subcutaneous fat relative to body weight, as anticipated due to the fat supplement in the other experimental groups. The levels of abdominal fat were lowest in the control group, while the 6%FA group recorded the highest values during both the grower and starter phases (Table 3). No significant differences were observed among the groups regarding the development of pectoral muscles during the starter phase. However, with the introduction of fat supplements during the grower phase, there was a noticeable improvement in pectoral muscle mass. This was evident as the control group exhibited the lowest measurement during this stage (Table 3).

Although serum total cholesterol levels remained constant among the experimental groups, the inclusion of a 6% fat supplement, regardless of its origin, increased serum triglyceride levels during both phases.

3.2 Gene Expression

Genes associated with the synthesis of fatty acids, such as FASN, SCD, and ME1, were examined in relation to hepatic fatty acid synthesis. The presence of fat, regardless of its source, notably enhanced the mRNA levels of these genes in hepatocytes. Moreover, the dietary fat content was found to upregulate FASN expression in the liver of the experimental chickens. While the amount of fat supplementation did not consistently impact SCD and ME1 expression, it was observed that SCD expression increased during the starter phase based on fatty acid levels, and fat powder levels influenced ME1 expression during the grower phase (Figure 2).



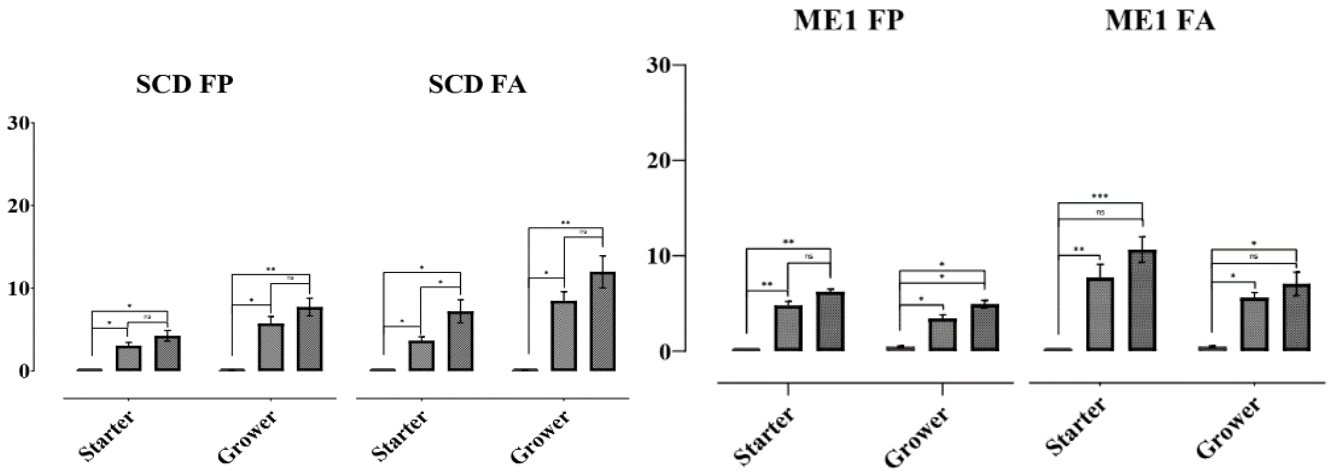


Figure 2. Fold Change of fatty acid synthesis-related genes in the liver of broiler chickens fed experimental diets (FP= fat powder; FA= fatty acids) at two levels (3% and 6%). * = The differences are significant at $p \leq 0.05$; ** = The differences significant at $p \leq 0.01$; *** = The differences significant at $p \leq 0.001$; ns = no significant difference. FASN=fatty acid synthase; ME1=malic enzyme 1; SCD=stearoyl-CoA desaturase. The error bars show SEM.

Between the chosen genes, GPAT3 and GPAM, associated with triglyceride synthesis, the mRNA abundance of these two genes was increased by fat supplements from both sources, as depicted in Figure 3. The fat inclusion level in most measurements was found to be a significant stimulator of genes related to triglyceride synthesis. Interestingly, during the starter phase, the expression of GPAT3 was downregulated by 3%FP. However, no changes were observed in GPAT3 and GPAM expression with respect to 3% FA or FP during the starter phase, respectively. It is worth noting that the inclusion of FA was more effective in upregulating genes related to triglyceride synthesis compared to FP in the current study (Figure 3).

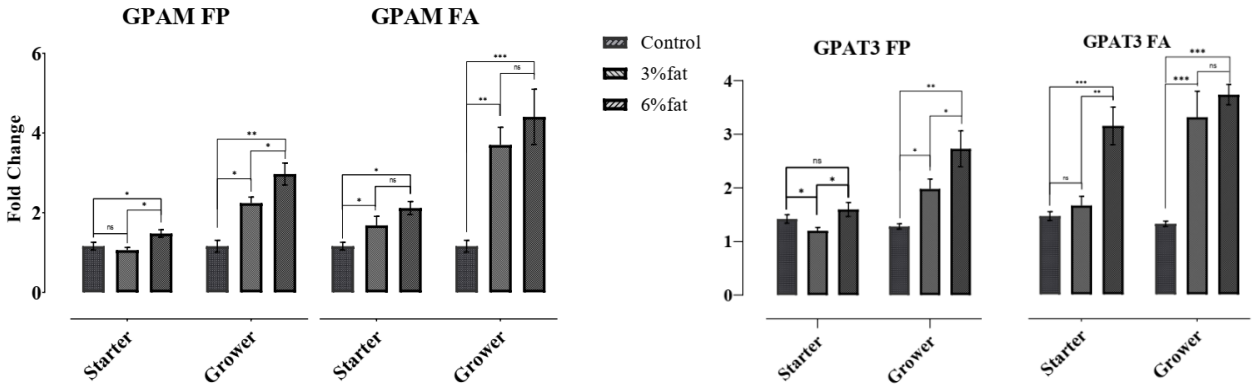


Figure 3. Fold Change of triglyceride synthesis-related genes in the liver of broiler chickens fed experimental diets (FP= fat powder; FA= fatty acids) at two levels (3% and 6%). * = The differences are significant at $p \leq 0.05$; ** = The differences significant at $p \leq 0.01$; *** = The differences significant at $p \leq 0.001$; ns = no significant difference. GPAT3=glycerol-3-phosphate acyltransferase 3; GPAM=glycerol-3-phosphate acyltransferase, mitochondrial. The error bars show SEM.

CD36, CPT1A, CPT2, and ACOX1 have been identified as genes associated with fatty acid oxidation. Notably, the results depicted in Figure 4 indicate that these genes did not show any significant stimulation in response to the experimental treatments administered in the initial phase. However, during the subsequent grower phase, it was noted that the mRNA levels of these genes increased when fat supplements were introduced. The influence of fat levels on the expression of genes related to fatty acid oxidation was generally minimal, except for CD36 (in both fat sources) and

CPT1A and CPT2, which were affected by the fatty acid levels (Figure 4).

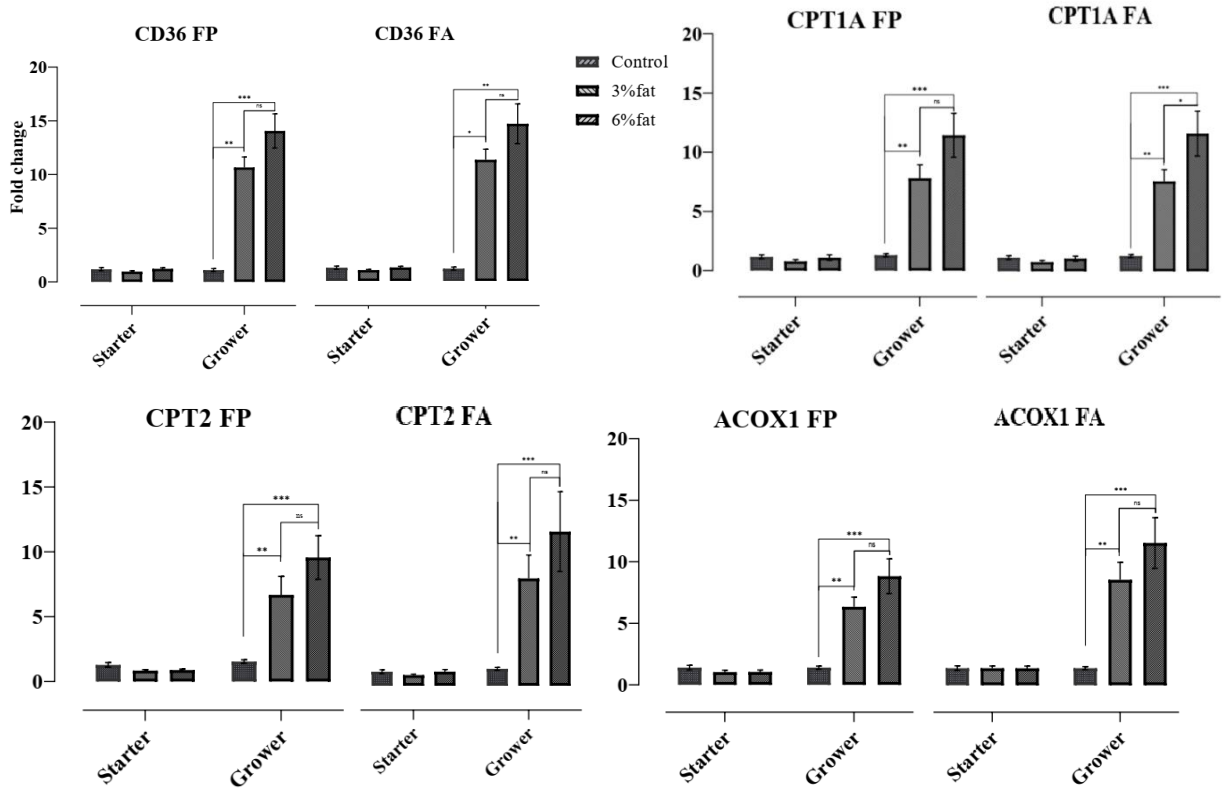


Figure 4. Fold Change of fatty acid oxidation-related genes in the liver of broiler chickens fed experimental diets (FP= fat powder; FA= fatty acids) at two levels (3% and 6%). * = The differences are significant at $p \leq 0.05$; ** = The differences significant at $p \leq 0.01$; *** = The differences significant at $p \leq 0.001$; ns = no significant difference. CD36= CD36 molecule; CPT1A=carnitine palmitoyl transferase 1A; CPT2=carnitine palmitoyl transferase 2; ACOX1=acyl-CoA oxidase 1; receptor gamma. The error bars show SEM.

The triglyceride transporting-related gene in liver APOB and apoVLDLII was examined in the current investigation. As depicted in Figure 5, the introduction of fat powder during the starter phase did not result in any noticeable changes in the expression of APOB. Nevertheless, during the grower phase, it led to a fivefold increase in the gene's expression, irrespective of the supplementation level. Conversely, the FA supplement itself upregulated the expression of APOB, regardless of the supplementation level. Notably, the FA supplementation enhanced APOB expression more efficiently than the fat powder.

Similarly, the presence of fat in the chicken diets impacted the expression of apoVLDLII, another gene involved in triglyceride transport. Both APOB and apoVLDLII were more effectively upregulated by FA than fat powder. However, it is crucial to note that only during the starter stage did the level of fat supplementation significantly influence the expression of apoVLDLII (Figure 5).

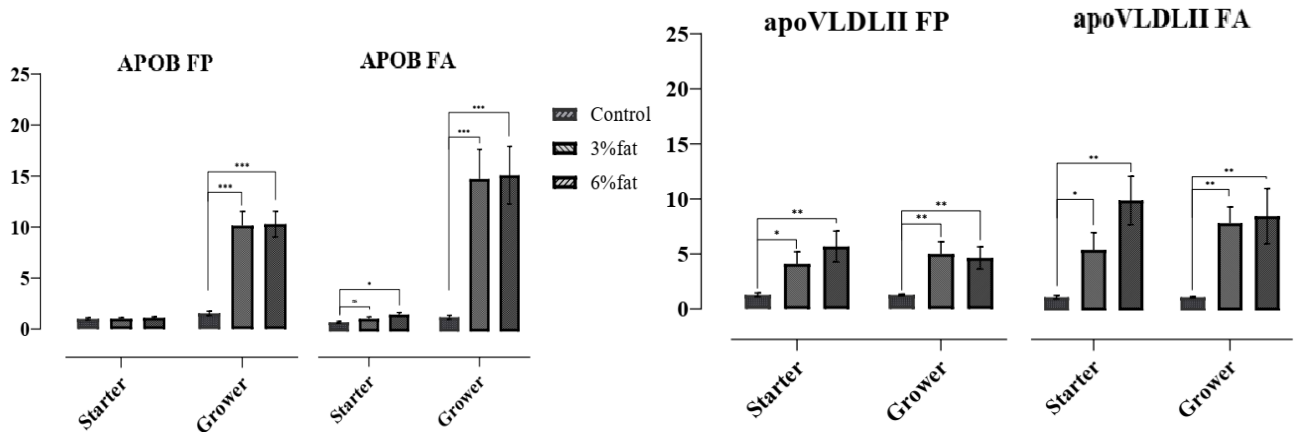


Figure 5. Fold Change of triglyceride transport-related genes in the liver of broiler chickens fed experimental diets (FP= fat powder; FA= fatty acids) at two levels (3% and 6%). * = The differences are significant at $p \leq 0.05$; ** = The differences significant at $p \leq 0.01$; *** = The differences significant at $p \leq 0.001$; ns = no significant difference. APOB=apolipoprotein B; apoVLDLII=very low-density apolipoprotein II. The error bars show SEM.

Among the selected genes, PPARα and PPARγ were identified as transcription factors associated with fat metabolism. Figure 6 illustrates that both fat sources significantly impacted these genes, with fatty acids exhibiting greater efficacy. Specifically, the expression of PPARα decreased during the initial phase when 3% FP was administered, while 6% FP and FA had no effect. However, in the subsequent growth phase, PPARα was upregulated approximately six times and 15 times by FP and FA supplements, respectively. PPARγ also exhibited a slight variation in response to fat supplements. Interestingly, the expression of this gene was significantly affected only by 6% of both fat supplements during both growth phases. Similar to other genes examined, FA was found to be more effective than FP in regulating PPARα and PPARγ (Figure 6).

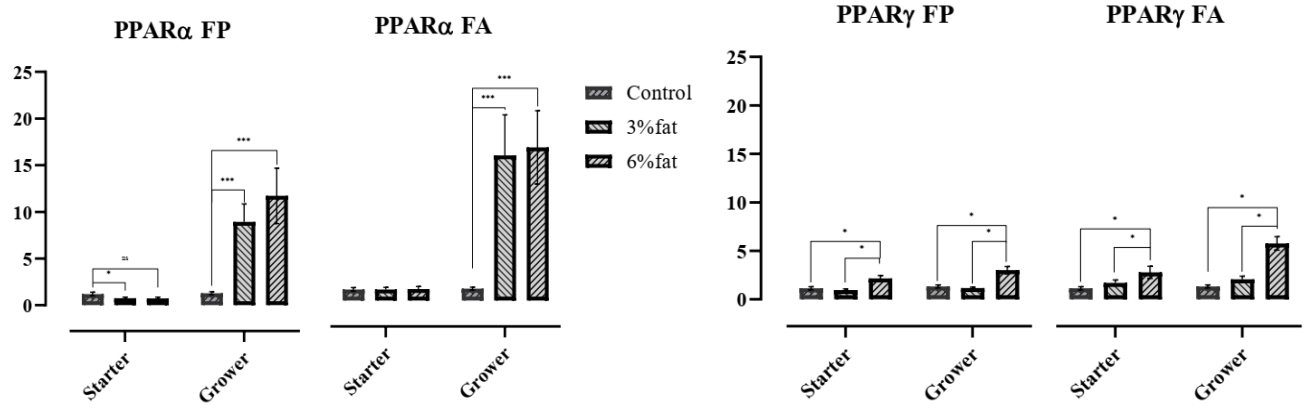


Figure 6. Fold Change of transcription factor genes in the liver of broiler chickens fed experimental diets (FP= fat powder; FA= fatty acids) at two levels (3% and 6%). * = The differences are significant at $p \leq 0.05$; ** = The differences significant at $p \leq 0.01$; *** = The differences significant at $p \leq 0.001$; ns = no significant difference. PPARα=peroxisome proliferator-activated receptor alpha; PPARγ=peroxisome proliferator-activated. The error bars show SEM.

4 Discussion

In the past few years, there has been an increasing fascination with comprehending the consequences of various sources of dietary fat on genes associated with hepatic fat in broiler chickens (25). This subject holds significance for the poultry sector and human well-being,

given that broiler chickens are widely consumed by individuals globally (26). Same researchers have emphasized the rapid advancement in this particular field of study, as distinguished experts have made significant contributions to our understanding of how different sources of dietary fat can impact the activation of genes associated with fat metabolism in the liver of broiler chickens (26). Several decades ago, early studies began to investigate dietary fats' significance in broiler chickens' growth and development. These studies aimed to identify the most suitable fat sources that promote healthy growth while minimizing the risk of diseases associated with excessive fat accumulation in the liver (20). Noteworthy researchers utilize cutting-edge molecular techniques like RNA sequencing and gene editing to delve deeper into the specific genes and pathways influenced by different dietary fats. This newfound knowledge may pave the way for tailoring personalized dietary recommendations for broiler chickens based on their genetic composition and uncovering innovative feed additives capable of modulating gene expression in the liver to enhance optimal fat metabolism.

The relationship between dietary fats and gene regulation in the liver of broiler chickens was elucidated by various pieces of evidence. Recent studies have shown that distinct types of dietary fats exert differential effects on hepatic fat metabolism, suggesting that specific fats play a more beneficial role in modulating the expression of key genes involved in fat storage and utilization within the liver (27).

The impact of specific fatty acids on the regulation of hepatic fat-related gene expression in broiler chickens has been the focus of research conducted by Kalia et al. (2023) and Pirany et al. (2020) (28, 29). Their investigations have discovered noteworthy findings that indicate the positive influence of certain fatty acids, such as omega-3 fatty acids, on the expression of genes involved in lipid metabolism within the liver. Conversely, saturated fats have been found to have detrimental effects. These findings hold significant implications for developing broiler chicken diets to enhance liver health and reduce the prevalence of fatty liver disease.

Our findings align with previously published evidence, confirming the potential of fat supplements to improve and regulate the fat metabolism process in the liver at a molecular level. The current results further solidify this notion. Additionally, our investigation revealed that different fat supplements exhibited varying degrees of effectiveness on the selected genes related to fat metabolism, consistent with prior research.

Moreover, our research findings could have significant implications for human well-being as they can inform dietary recommendations to reduce the risk of liver diseases associated with excessive fat accumulation. On a positive note, the investigation focusing on the influence of different sources of dietary fat on genes related to hepatic fat in broiler chickens has the potential to improve the overall health and welfare of these chickens, leading to a more sustainable and efficient poultry production system. By carefully selecting dietary fat sources to regulate gene expression in the liver, farmers can promote optimal growth and minimize the occurrence of fatty liver disease among their chicken populations. This approach has been well reviewed by previous publications by Fouad and El-Senousey (2014), Pirany et al. (2020), and Nematbakhsh et al. (2021) (27, 29, 30).

Conversely, there are adverse implications regarding the influence of various dietary fat origins on hepatic fat-related genes in broiler chickens. Specifically, some dietary fats that stimulate the excessive expression of genes linked to fat accumulation in the liver may result in heightened fat accumulation and diminished meat quality in broiler chickens (27, 29, 30). Furthermore, incorporating certain fats into poultry diets could have harmful environmental consequences, including contributing to deforestation or greenhouse gas emissions related to feed ingredient production (31).

Fatty acid synthase (FASN) is crucial in synthesizing fatty acids from acetyl-CoA and malonyl-CoA. It plays a pivotal role in lipogenesis, converting surplus carbohydrates into fatty acids for storage (25). In a study conducted by Jeun-Horng el al. (2002), the impact of dietary fat supplements on FASN expression in the liver of broiler chickens was examined (32). The results indicated that adding fish oil led to a significant decrease in FASN mRNA expression compared to chickens on a standard diet. In the current investigation, it was noted that the addition of FA or FP to the diet resulted in elevated levels of plasma TG. It is widely accepted that dietary fat is absorbed into the portal blood system, with blood lipids primarily entering hepatic tissue for processing (33). The hepatic mRNA levels of PPARγ, SCD, ME1, and FASN were assessed to determine the liver's capability for fatty acid synthesis. The elevated mRNA levels of PPARγ, SCD, ME1, and FASN in the treatments could be due to the increased need for lipids in the blood, indicating enhanced fatty acid synthesis in the liver. Although not widely recognized, fat-fed chickens' higher abdominal adipose tissue may be linked to the higher

mRNA expression of the selected genes involved in hepatic lipogenesis. In avian species, the liver is the primary site for fatty acid synthesis, with subsequent transport and storage of TG in adipose tissues (34). This could explain the higher serum TG levels observed in our study.

Stearoyl-CoA desaturase is a key enzyme in fatty acid metabolism, specifically in converting saturated fatty acids to monounsaturated fatty acids. It plays a critical role in regulating lipid composition and is thought to be a significant factor in the development of metabolic disorders such as obesity and insulin resistance (25, 27). A study by Shang et al. (2005) investigated the influence of dietary fat supplementation on SCD expression in the liver of laying hens (35). The research demonstrated that including linseed oil significantly elevated SCD mRNA expression compared to a standard diet. The higher intake of lipids and saturated fatty acids in the study may have increased the demand on hepatocytes to promote desaturation, leading to the upregulation of SCD expression. The elevated level of saturated fatty acids (SFA) in the diet increases the overall cholesterol levels in the bloodstream, including LDL-cholesterol and HDL-cholesterol. This, in turn, contributes to the development of coronary artery disease (36).

On the other hand, a higher enzymatic activity of stearoyl-CoA desaturase or SCD abundance in hepatocytes can be seen as advantageous for individuals consuming broiler products, as it reduces the maturity index of these lipids. Further research is warranted to investigate additional aspects, such as post-translational studies related to changes in SCD and desaturation capacity and the qualifying process. These remaining issues necessitate further in-depth investigations.

ME1, also called malic enzyme 1, is a vital enzyme that plays a crucial role in converting malate to pyruvate. This enzymatic reaction is a pivotal step in the metabolic pathway that leads to the synthesis of NADPH. This cofactor has a significant impact on the synthesis of fatty acids. ME1 actively participates in lipogenesis and maintains the delicate equilibrium between glucose and fatty acid metabolism (37). In a recent investigation by Leng et al. in 2019, the researchers aimed to examine the influence of dietary fat supplementation on the expression of ME1 in the liver of broiler chickens. Specifically, they explored the effects of adding coconut oil to the diet and its impact on the expression of ME1 mRNA, comparing it to a control diet. Surprisingly, the results demonstrated that adding coconut oil significantly decreased the expression of ME1 mRNA when compared to the control diet. The disparity between our findings and those of Ahmadipour et al. (2019) could be attributed to variations in the fat content and composition of the basal diets and the sources of fat used in the studies (38). Wang and colleagues (2021) demonstrated that the potential fat regulation effect could be influenced by the fatty acids composition of the supplemented fat (39). The chickens in the fat-included experimental groups exhibited an elevation of circulatory TG content by over 80 percent compared to the control group. While not definitive, this rise may be linked to the activation of genes related to fatty acid synthesis, including ME1, which warrants additional research.

Numerous investigations have indicated that adding dietary fat can influence the regulation of genes related to lipid metabolism in the liver. Wang et al. (2021) conducted a study in which broiler chickens were provided with diets containing various fat sources such as soybean oil, palm oil, and coconut oil (39). The outcomes revealed a significant impact of the type of fat supplement on the expression of GPAT3 and GPAM genes in the liver. Specifically, the expression of GPAT3 was increased in chickens that consumed the soybean oil diet compared to those on the palm oil and coconut oil diets. Conversely, the expression of GPAM was decreased in chickens fed the palm oil diet compared to those on the soybean and coconut oil diets. These results indicate that different dietary fats can lead to distinct effects on gene expression related to lipid metabolism in the liver of chickens. Polyunsaturated fatty acids in soybean oil have been shown to activate transcription factors such as peroxisome proliferator-activated receptors (PPARs) that regulate the expression of genes involved in lipid metabolism (40).

Conversely, saturated fats like those in palm oil have been demonstrated to induce inflammation and insulin resistance, potentially influencing gene expression in the liver (41). Furthermore, the control of GPAT3 and GPAM expression by dietary fats may also be impacted by feedback mechanisms in lipid metabolism. For instance, the increased synthesis of triglycerides from fatty acids could inhibit the expression of GPAM, which is responsible for glycerol-3-phosphate synthesis, a key precursor for triglyceride formation (42). Conversely, the upregulation of GPAT3, the enzyme initiating triglyceride synthesis, may lead to triglyceride accumulation in response to a high-fat diet (43). Our research results align with the previously published literature. The elevation of GPAT3 and GPAM could be associated with increased fat content and higher levels of polyunsaturated fatty acids in the experimental diets. Further

and extensive research is required to understand these genes' regulatory mechanisms comprehensively.

CD36, also known as fatty acid translocase, is a protein integrated into the membrane that plays a role in the absorption and storage of fatty acids in the liver (44). Studies have demonstrated that introducing fat supplements can upregulate the expression of CD36, CPT1A, CPT2, and ACOX1 in chicken liver, leading to increased uptake of fatty acids (45-47). This process may help prevent excessive fat accumulation in the liver and promote energy utilization. It could potentially result in a more efficient utilization of dietary fats and a reduction in lipid buildup in the liver.

In a study by Zhang et al. (2016), broilers fed a diet enriched with 3% soybean oil exhibited significantly higher body weight than those fed a control diet (48). This finding suggests that including fat supplements in broiler diets can enhance their growth performance by providing additional energy for growth and maintenance. Also, dietary fat consumption reduced the passage rate at which the digest traversed the gastrointestinal tract, resulting in enhanced absorption and utilization of nutrients (49).

Apart from body weight, the deposition of abdominal fat is another crucial aspect of broiler production. Excessive accumulation of abdominal fat can negatively impact broilers' feed efficiency and meat quality. Jiang et al. (2019) conducted a study investigating fat supplements' effects on broilers' abdominal fat (50). The results revealed that broilers fed a diet supplemented with 5% fish oil experienced a significant reduction in abdominal fat compared to those fed a control diet (50). The current findings also approve the published data. This indicates that incorporating fat supplements in broiler diets can aid in reducing abdominal fat deposition, potentially leading to improved feed efficiency and meat quality. Research has indicated that fat supplements can significantly influence broilers' serum cholesterol and triglyceride levels. For instance, a study by Sola-Ojo (2019) demonstrated that incorporating palm oil into broilers' diets resulted in a notable rise in serum cholesterol levels (51). This elevation in cholesterol levels can be linked to the high saturated fat content in palm oil, a known factor in increasing cholesterol levels in humans and animals.

Conversely, a separate study by Saleh et al. (2021) revealed that including soybean oil in broilers' diets reduced serum triglyceride levels (52). This decline in triglyceride levels can be attributed to the high unsaturated fat content found in soybean oil, which has been proven to impact lipid metabolism positively. These two studies underscore the divergent effects of various fat supplements on serum cholesterol and triglyceride levels in broilers. While palm oil may result in heightened cholesterol levels, soybean oil could benefit triglyceride levels. It is crucial for broiler producers to thoughtfully consider the type of fat supplement they incorporate into their birds' diets to attain optimal growth performance and overall well-being.

Our findings indicate that chickens possess gene regulatory pathways for fatty acid oxidation that resemble those observed in mammals. Fatty acid oxidation is a metabolic process that breaks down fatty acids to generate energy in fasting mammals. This process is controlled by the transcription factor PPARα, leading to reduced fat accumulation (53, 54). The expression of PPARα is significantly associated with FAT, CPT1A, CPT2, and ACOX1 expression in our study. While mice lacking Ppara exhibit impaired fatty acid oxidation and develop fatty liver, an increase in Ppara expression has been noted in mouse models of obesity (55). Therefore, it is evident that chickens have genetic mechanisms that regulate fatty acid oxidation like that seen in mammals.

PPARγ, a crucial transcription factor responsible for fat accumulation, is predominantly expressed in adipose tissues. It plays a significant role in promoting adipose differentiation in both rodents (56) and chickens (57, 58). Recent studies have also highlighted the involvement of PPARγ in hepatic lipid metabolism in mammals (53, 54). Memon et al. (2000) conducted an experiment on obese mice with elevated hepatic levels of PPARγ and observed that the expression of CD36, a downstream target of PPARγ, was induced in the liver but not in adipose tissue after treatment with a PPARγ agonist (59). This finding suggests that the activation of PPARγ expression in chickens, similar to rodents, may have triggered the up-regulation of CD36 expression, thereby facilitating fatty acid uptake.

Yang et al. (2017) conducted a study that revealed that broilers consuming a diet containing 5% soybean oil exhibited a lower FCR than those on a fat-free diet (60). This enhancement in FCR can be attributed to the heightened energy density of the diet, enabling birds to fulfill their energy needs more effectively. Moreover, fat supplements can boost nutrient absorption and utilization in broilers. Incorporating fat in the diet can enhance the digestibility of various nutrients, including protein and amino acids, resulting in improved growth performance and FCR. This was evidenced in a study by Jalili and Nobakht (2017), where broilers fed a diet supplemented with 2% sunflower oil displayed enhanced nutrient digestibility and FCR

compared to those on a standard diet (61). The fatty acid profiles greatly influence the digestibility of dietary fats. Several studies have provided evidence that unsaturated fats are utilized more effectively, leading to a higher metabolizable energy when compared to saturated fats (49). Lower FCR also relates to lower feed intake.

Various factors can contribute to this decline. Firstly, the presence of FP can impact the appearance, scent, and fragrance of the feed, thereby altering its palatability. Aprianto et al. (2023) discussed Ca-soaps' impact on feed quality, highlighting that the brownish and darker hue of Ca-soaps can alter the taste and aroma of the feed. Chickens can distinguish different feed colors, affecting their consumption behavior (36). Furthermore, transforming liquid oil into flour in the form of calcium salt can lead to a dustier texture in the feed. The researchers observed that calcium soap derived from long-chain fatty acids may reduce feed acceptability for livestock (36). The flavor and scent of calcium soap can reduce the palatability of the feed, resulting in decreased intake. Moreover, the dusty nature of the feed can irritate the nasal passages and eyes of animals. Additionally, the sour taste imparted by calcium soap can have a negative impact on palatability, leading to reduced feed intake and performance (36).

Ghobashy et al. (2023) conducted a study that revealed that broiler chickens provided with a diet enriched with soybean oil exhibited notably increased pectoral muscle weight compared to those given a standard diet (62). Soybean oil is abundant in essential fatty acids like linoleic acid, facilitating muscle growth and development in broiler chickens. These fatty acids in soybean oil can trigger protein synthesis and boost muscle cell proliferation, ultimately augmenting pectoral muscle weight. Saminathan et al. (2022) conducted a study comparing the impact of soybean oil and palm oil on the pectoral muscle weight of broiler chickens, and discovered that chickens fed a diet supplemented with soybean oil had significantly higher pectoral muscle weight compared to those fed palm oil (63). This disparity could be attributed to the variation in fatty acid composition between the two sources, as soybean oil contains elevated levels of essential fatty acids that support muscle growth. It is important to highlight that the pectoralis major muscle in modern broilers has significantly increased in size, being around eight times larger than those found in broilers from the mid-1950s. This notable growth results from intentional breeding for improved pectoralis major muscle development, making up 8-11% of the birds' total body weight. This trend has been primarily influenced by consumer preferences for a budget-friendly, lean protein choice (64).

Excessive accumulation of abdominal fat poses a significant challenge in the broiler industry, hindering the progress of modern broiler and layer production. However, it is crucial to emphasize that subcutaneous adipose tissue, which develops before abdominal adipose tissue, serves as a reservoir for releasing free fatty acids after hatching and heat generation during the starter phase (65). Nevertheless, in finishing broiler chickens, the increase in subcutaneous adipose tissue contributes to the overall fat content of the consumable carcass, potentially posing a risk to public health. Incorporating vegetable oil into the diets of broiler chickens has been found to elevate the level of subcutaneous fat in these birds, as indicated by various studies. For instance, Wang et al. (2017) conducted a study that revealed a significant increase in subcutaneous fat among broiler chickens fed a diet supplemented with vegetable oil in comparison to those fed a control diet (66). This rise in subcutaneous fat can have both advantageous and disadvantageous consequences for poultry producers. On the one hand, higher subcutaneous fat levels can enhance the meat's flavor and succulence, thereby leading to heightened consumer satisfaction.

Conversely, excessive subcutaneous fat can impede the overall efficiency of poultry production by reducing the meat yield per bird. In contrast to vegetable oil, animal fat is another commonly used fat supplement source in broiler chicken diets. Animal fat is a highly concentrated energy source that birds can more readily metabolize than vegetable oil. Studies have also demonstrated that incorporating animal fat into broiler chicken diets can elevate the level of subcutaneous fat in these birds. For example, Ferrini et al. (2008) conducted a study that found that broiler chickens fed a diet supplemented with animal fat exhibited a higher subcutaneous fat level than those fed a control diet (67). The rise in subcutaneous fat can have comparable consequences for poultry farmers, as seen with the addition of vegetable oil, which impacts both the quality of meat and the efficiency of production. Conversely, since subcutaneous adipose tissue is deemed crucial during the initial stages of a newly hatched chicken's life, as well as during the starter period (65), it is imperative to conduct extensive and meticulous research to discover a technique that can regulate the growth of this tissue throughout the lifespan of a broiler chicken.

5 Discussion

Understanding the impact of fat supplements on gene expression in poultry presents a promising opportunity to enhance growth performance and health outcomes. Through strategic manipulation of fat sources and levels in the diet, producers can improve feed efficiency, reduce production costs, and elevate the quality of poultry products. This knowledge also opens doors for creating specialized diets tailored to meet the unique nutritional requirements of various poultry breeds and production systems. The inclusion of fatty acids (FA) exhibited a more potent regulatory effect on the genes under investigation. Nevertheless, there are adverse implications associated with using fat supplements in poultry diets. High-fat diets may accumulate abdominal fat, potentially compromising carcass quality and consumer acceptance of poultry products.

Additionally, excessive fat consumption can trigger metabolic disorders and health complications in chickens, detrimentally affecting their overall welfare. Producers must thoroughly evaluate the impact of fat supplements on gene expression and metabolic pathways to mitigate potential drawbacks in poultry production. Although the fat level was a significant factor in some instances, based on the current findings and methodology, a 3% fat supplement may be more cost-effective and economical for farm use. Future investigations in the realm of fat metabolism in poultry should prioritize conducting comparative and correlative analyses encompassing a broader array of fat sources while also considering additional production traits in conjunction with genes linked to fatty acids in the liver. By broadening the scope of these studies, researchers can attain a more holistic comprehension of the fat metabolism process in poultry and uncover innovative strategies to enhance growth performance and health outcomes. Furthermore, advancements in molecular techniques like RNA sequencing and gene editing hold promise in unraveling the intricate mechanisms through which fat supplements influence gene expression in poultry, thereby paving the way for groundbreaking solutions in poultry nutrition and management.

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Conflict of Interest

The authors have no conflict of interest to be declared.

Author Contributions

All authors contributed equally in this work.

Data Availability Statement

The datasets generated during and analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Ethical Considerations

The Yasouj University, Iran, approved all procedures conducted in this study after a thorough evaluation by the Institutional Animal Care and Use Committee under license 229707331.

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