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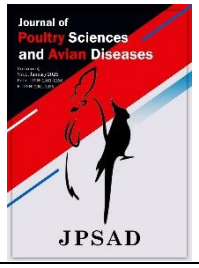

Exploring Performance and Hepatic Lipid Metabolism in Broilers: The Influence of Lysophospholipid Supplement Levels and Dietary Fat Sources in Diets

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A B S T R A C T

The aim of this investigation was to assess the influence of integrating a lysophospholipid (LPL) nutritional supplement with fat on the constitution of blood serum lipids, the microbial population in the cecum, and the expression of genes involved in controlling liver fat metabolism in juvenile chickens. A factorial complete randomized design was utilized, encompassing 1440 one-day-old broiler chickens of the Ross 308 variety. There were 12 treatments and 12 replications for each treatment. Blood and liver specimens were gathered for examination. The outcomes of the study demonstrated that the addition of LPL and various levels of fat had a notable impact on the levels of triglycerides in the blood serum, along with the expression of genes linked to liver fat metabolism ($P \leq 0.05$). Furthermore, the administration of LPL led to an increase in the population of Lactobacillus bacteria within the cecum of the examined avian species ($P \leq 0.05$). Nevertheless, no significant effects on overall performance were detected ($P > 0.05$). This investigation underscores the significance of lysophospholipids in poultry nutrition and liver function, and proposes the necessity for further exploration into diverse genes and sources of fat. Comprehending the gender-specific variances in lipid metabolism pathways is essential for upholding the health and productivity of poultry. These discoveries establish a solid groundwork for enhancing poultry nutrition and enhancing production efficiency to fulfill the economic requirements of producers and the health needs of consumers.

Keywords: *Hepatocyte Steatosis, Hepatic Gene Expression, Lipid Metabolism, Lysophospholipid, Supplementary Fat*

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1 Introduction

Given the significant proportion of expenses in poultry farming dedicated to feed, meticulous adjustment of feed composition is crucial. Corn (*Zea mays*) remains a primary energy source, but exploring alternative cost-effective energy-generating feeds is essential due to rising corn seed prices (1-3). Fat supplementation boosts energy provision due to its higher energy density (2). The digestion and absorption of dietary fats involve emulsification by bile and hydrolysis by pancreatic lipase, converting triglycerides into absorbable monoglycerides and free fatty acids (2). The inclusion of fat in poultry feed aids in energy utilization and contributes to the overall yield of poultry industry. Numerous studies have provided evidence that the capacity of young chickens to process and assimilate lipids is limited and is associated with their physiological development, inadequate production of lipase (2, 4), the condition of their intestinal microflora, the composition of their diet (5), the ratio of unsaturated fatty acids to saturated ones in their diet (6), the presence of pentosans (7), and the inclusion of dietary fiber (8).

The optimal digestion and absorption of nutrients play a vital role in poultry production, given the limited capacity of their digestive system (9). In the case of young birds, the digestion of fats is not as efficient due to inadequate secretion of lipase and bile acids, resulting in incomplete digestion and absorption (2, 9). Consequently, diets may not supply sufficient nutrients, impeding growth and productivity. Hence, it is advisable to include emulsifiers as additives to enhance fat digestion and increase energy yield in a smaller quantity, thereby enhancing the overall performance of poultry (10).

An emulsifier is a molecule that possesses both hydrophilic (water-soluble) and lipophilic (fat-soluble) properties. This unique characteristic allows the molecule to dissolve in water through its hydrophilic part and in fat through its hydrophobic part. By doing so, emulsifiers play a vital role in the formation of micelles (11). These emulsifiers aid in the distribution of fat droplets within emulsions, which is crucial for the digestion and absorption of fats. In the case of poultry, LPLs serve as effective emulsifiers for fat digestion and absorption. These natural surfactants are derived from the hydrolysis of soy lecithin by phospholipase A_2 (12). Lysophospholipids have a hydrophilic-lipophilic balance ranging from 2 to 12, which is higher than that of bile salts and lecithin. Additionally, LPLs have a lower critical micelle concentration compared to bile salts and lecithin, resulting in the formation of smaller micelles in the bird's intestine and an increased surface area for fat droplets (13).

Incorporating fat and LPL into animal feed formulations has enabled producers to decrease their dependence on costly cereal grains, leading to reduced feed expenses and enhanced financial gains (11). Compounds that enhance nutrient digestion in the gastrointestinal tract are utilized to improve performance by modifying the metabolism of key nutrients (9, 11). Gaining a comprehensive understanding of the metabolic mechanisms underlying these changes can facilitate the optimization of processes, enhance productivity, and ultimately improve the economic performance of poultry farming operations. The aim of this study was to examine the impact of incorporating a LPL nutritional additive in conjunction with fat on the composition of blood serum lipids, cecum microbial population, and the expression of genes responsible for regulating liver fat metabolism in young chickens. Furthermore, the research assessed the effectiveness of dietary fat breakdown when LPL was present.

2 Methods and Materials

2.1 Chickens, housing, feed and management

The Yasouj University, Iran, granted approval for all procedures conducted in this study after thorough evaluation by the Institutional Animal Care and Use Committee under the license 229707331. The research was conducted at the broiler farm owned by Sabzbavaran-e-Nouandish Company, which is located on Firozabad-Qirokarzin road in Fars province, Iran. The corresponding analyses were carried out in the animal feeding laboratory of the same company. Refined safflower oil, produced by a local commercial company, was obtained from a human food store. A quantity of 50 kg of cow tallow was acquired from Shiraz industrial slaughterhouse. In order to incorporate the tallow into the experimental rations, it underwent a series of steps. Initially, the tallow was ground after removing any impurities, and then it was melted using the indirect heat of a microwave oven at an average temperature of 100°C. After passing through a 0.1 mm sieve, the melted tallow was collected and stored at a temperature of 4°C until it was used in the rations. The supplement Artefier, a LPL product developed by Artevet LLC., Wilmington, Delaware, contained polyethylene glycol ricinolate, Lysophosphatidylcholine, Lysophosphatidylethanolamine, Lysophosphatidylinositol, and lysophosphatidic acid.

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

Item	1.5 % supplement			3% supplement		
	SO*	BT*	SO+BT*	SO*	BT*	SO+BT*
Soybean meal (47% CP)	37.6	37.04	37.19	38.47	38.48	38.46
Corn (8.3% CP)	55.84	56.4	56.25	53.43	53.42	53.44
Soy bean oil	1.50		0.75	3.00		1.50
Tallow)		1.50	0.75		3.00	1.50
DL-Met	0.21	0.21	0.21	0.21	0.21	0.21
L-Lys HCl	0.18	0.18	0.18	0.18	0.18	0.18
L-Thr	0.11	0.11	0.11	0.11	0.11	0.11
Salt	1.60	1.60	1.60	1.60	1.60	1.60
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	2900	2900	2900
CP	20.84	20.84	20.84	20.84	20.84	20.84
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	1.72	1.37	1.41	1.54	1.87	122
Calcium	1.00	1.00	1.00	1.00	1.00	1.00
Available phosphorus	0.45	0.45	0.45	0.45	0.45	0.45
Sodium	0.20	0.20	0.20	0.20	0.20	0.20
Analyzed nutrient level, %						
Total lipid, %	6.71	6.20	6.51	8.10	8.41	8.82
Fatty acid profile (% of total fatty acid)						
SFA[3]	28.96	45.82	37.39	15.30	57.40	36.35
MUFA[4]	20.01	48.16	34.09	23.80	37.20	30.50
PUFA[5]	51.03	6.02	28.52	60.90	5.40	33.15
SFA[3]/MUFA[4]	1.45	0.95	1.10	0.64	1.54	1.19
n-6[6]	47.06	5.18	21.97	54.10	3.11	28.62
n-3[7]	0.52	0.92	0.72	0.41	0.60	0.52
n-3/n-6 ratio[8]	0.01	0.18	0.03	0.01	0.19	0.02

* SO = Safflower oil; BT = Beef tallow; SO+BF = 50% safflower oil+50% beef tallow.
** The six diets were partitioned into two equal parts, and then 0.025% LPL was incorporated into one of the portions. Subsequently, the mixtures were combined to create a total of 12 diets.
[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-3 to omega-6 polyunsaturated fatty acid ratio.

A factorial complete randomized design was employed in this study, utilizing a total of 1440 one-day-old broiler chickens of the Ross 308 strain. The design consisted of two levels of LPL supplementation (0 and 0.25 g/kg), two levels of fat (1.5 and 3%), and three different fat sources (safflower oil, cow tallow, and a 50:50 mixture). There were 12 treatments in total, each with 12 replicates (6 male and 6 female), and 10 chickens per replicate. The diets were carefully formulated to be isocaloric and isonitrogenous, following the nutritional requirements recommended by the national research council – NRC 1994 (14), as presented in Table 1. 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 (15). To ensure that all the chickens' nutritional needs were met, the UFFDA software was utilized to adjust the rations accordingly. The fat sources used in this study are detailed in Table 1. The experimental diets were administered to the chickens from one to 14 days of age, during the starter phase. It is worth noting that commercial poultry diets typically incorporate a fat addition ranging from 20 to 50 g/kg, which is determined by the prevailing prices of fat and cereal grains (16). Throughout the duration of the experiment, the mortality rate of the birds in each enclosure was recorded on a daily basis. In order to identify any potential lesions, necropsies were performed on the birds that perished during the course of the experiment.

2.2 *Sampling*

Following the conclusion of the 14-day experimental period, a total of 120 birds were selected based on their weights closely matching the average weight of their respective groups for additional assessment. Each bird chosen underwent exsanguination from the jugular vein to collect blood samples. The serum was isolated through centrifugation at 3000xg for 10 minutes at 4°C and then preserved at -20°C for future investigations. The left hepatic lobe was excised, flash-frozen in liquid nitrogen for a day, and subsequently moved to -70°C for further examination.

2.3 *Laboratory Analysis*

The measurement of plasma cholesterol and triglyceride concentrations was conducted with the commercial kit manufactured by Pars Azmoun in Karaj, Iran. On the other hand, the quantification of plasma HDL and LDL levels was carried out using the commercial kit produced by ZistShimi in Tehran, Iran, with the assistance of the autoanalyzer Biotechnica BT-3000 from Italy.

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, as previously described (17). The quality and quantity of the RNA were assessed using a NanoDrop (ND-100 UV-Vis; Nanodrop Technologies, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized using a high-performance cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) based on a known protocol. The primers for qRT-PCR were designed using software tools like Primer Express Software for Real-Time PCR version 3.0.1 (Thermo Fisher Scientific Inc.), Primer3Plus version 2.4.2, or Primer-BLAST. Specific primers utilized in this study are listed in Table 2. qRT-PCR was carried out on an ABI 7500 fast instrument (Applied Biosystems, ABI 7500 fast, CA, USA) with a reaction volume of 10 µL and SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio Inc.). The cycle threshold (CT) of the target genes was normalized to 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 various treatments.

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			

Gene symbol	Forward primer (5′-3′)	Reverse primer (5′-3′)	Accession no.
Fatty acid synthesis			
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			
PPARα	CAAACCAACCATCCTGACGAT	GGAGGTCAGCCATTTTTTGGA	NM_001001464
PPARγ	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; PPARα=peroxisome proliferator-activated receptor alpha; PPARγ=peroxisome proliferator-activated receptor gamma; 18SrRNA=18S ribosomal RNA.

2.5 Microbial study

To determine the microbial population of Lactobacillus and Escherichia coli in the cecum of slaughtered chickens, the cecums were extracted from the chickens at the end of the 14-day experiment. The contents of the cecums were either removed manually or by gentle finger movement. Subsequently, the cecum contents were collected in sterile containers, placed on ice, and then stored in a freezer at -20°C. For bacterial enumeration, one gram of cecum contents from each sample was diluted in nine milliliters of distilled water. Two dilutions (10^{-1} and 10^{-2}) were prepared from the resulting homogenous solution, and each dilution was plated on specific culture media. *Lactobacillus* and *Escherichia coli* were cultured on MRS and EMB agar plates, respectively. The plates were then incubated at 37°C under anaerobic and aerobic conditions for 48 hours and 24 hours, respectively, to allow for the growth of Lactobacillus and Escherichia coli colonies. Following the incubation period, bacterial colonies were enumerated using a Colony Counting device (18).

2.6 Statistical analysis

The data's normal distribution was verified through the utilization of the SAS UNIVARIATE method (version 9.4, SAS Institute Inc., Cary, NC, USA). To assess the data, the SAS mixed model procedure was utilized, with LPL (-, +), fat source (three sources), fat level (two levels), sex, and their interaction being considered as fixed effects. Chickens were treated as repeated subjects in the model. The SAS General Linear Model was used to test the ΔΔCTs' results. Mean comparisons were conducted using Tukey's method. A statistically significant discrepancy was identified when the probability level (P-value) fell under 0.05. Moreover, a tendency towards significance was noted when the difference in means had a probability level (P-value) ranging from 0.06 to 0.1.

3 Results and discussion

3.1 Effects on performance

The data presented in Table 3 illustrates the impact of LPL supplementation in diets with varying fat sources and levels on average body weight, body weight gain, feed consumption, and feed conversion ratio during the initial period. The findings of the analysis indicated that the combined effects of LPL supplementation with different fat sources and varying levels of dietary fat did not produce statistically significant outcomes, except for the feed consumption of broilers, which showed a significant difference (P=0.02). Specifically, chickens fed a diet lacking LPL supplementation and containing 1.5% fat exhibited the lowest feed consumption (P<0.05). Among the primary effects, only the fat level in the diet had a significant impact on average body weight and body weight gain (P<0.05). A fat level of 3% compared to 1.5% resulted in improved body weight and increased daily weight gain in broiler chickens (P=0.03). Interaction effect data were not presented due to lack of significance.

Table 3. Impact of lysophospholipid (LPL) on the growth outcomes of young chickens given varying amounts and sources of fat-rich diets in starter stage

Independent variable	Level or type	Live body weight (g)	Average gain (g)	Average feed intake(g)	Feed conversion ratio
LPL	0.025%	335.94	21.33	25.81	1.21
	0	331.56	20.93	25.12	1.20
	SEM	3.91	0.81	0.30	0.02
Fat source*	SO	331.86	20.86	24.82	1.19
	BT	329.92	20.59	24.91	1.21
	SO+BT	332.38	20.71	25.05	1.21
	SEM	4.16	0.47	0.46	0.03
Fat level	1.5%	328.71^{b}	20.11^{b}	24.73	1.23
	3%	341.43^{a}	22.06^{a}	26.69	1.21
	SEM	4.66	0.31	0.49	0.03
Sex	Male	348.13^{a}	23.19^{a}	29.68^{a}	1.28^{a}
	Female	314.40^{b}	20.14^{b}	23.36^{b}	1.16^{b}
	SEM	5.22	0.53	0.27	0.02
P-value					
LPL (L)		0.45	0.29	0.19	0.29
Fat source (F_S)		0.36	0.31	0.79	0.24
Fat level (F_L)		**0.03**	**0.04**	0.61	0.41
Sex (S)		**0.02**	**0.01**	**0.05**	**0.03**
L×F_S		0.52	0.61	0.77	0.31
L×F_L		0.13	0.21	0.16	0.29
L×S		0.08	0.09	0.31	0.42
F_S×F_L		0.76	0.82	0.79	0.50
F_S×S		0.48	0.67	0.31	0.22
F_L×S		**0.04**	**0.05**	0.07	0.26
L×F_S×F_L		0.29	0.17	0.69	0.62
L×F_S×S		0.34	0.79	0.56	0.20
L×F_L×S		0.17	0.12	0.41	0.48
F_S×F_L×S		0.69	0.33	**0.02**	0.42
L×F_S×F_L×S		0.52	0.62	0.59	0.26

$^{a–b}$ Significantly different treatments in the starter phase are denoted by superscript letters with different subscripts at a significance level of $P \leq 0.05$

* SO = Safflower oil; BT = Beef tallow; SO+BF = 50% safflower oil+50% beef tallow

Based on the findings of this investigation, it was observed that the inclusion of LPL supplement did not lead to any enhancement in the performance of broiler chickens ($P>0.05$). Polycarpo et al. (2016) noted that during the initial 1-14 days of age, there was no significant interaction between the various factors under scrutiny (such as the type of fat source, presence or absence of LPL, and organic acids supplement) with regards to the performance of broiler chickens (19). Similarly, Jamili et al. (2013) highlighted that across different rearing periods, the combined impact of fat source and emulsifier (bile salt and lecithin) did not yield any significant effects on performance-related traits, except for feed consumption during the initial phase (20). Furthermore, Zampiga et al. (2016) reported that the influence of emulsifier at different nutritional stages did not exhibit any statistically significant differences across all production parameters considered (21). In a similar vein, Zhao et al. (2015) did not observe any notable disparities in the production performance of piglets when fed diets containing varying concentrations of LPL-based emulsifier (22). Additionally, Abbas et al. (2016) found no significant interaction between fat and emulsifier during the initial and overall breeding periods in relation to the body weight of broiler chickens, although the use of emulsifier supplement did result in improved body weight during the final period (23). These results stand in contrast to the research conducted by Upadhaya and colleagues, (2018) who demonstrated that supplementing the diet with a specific emulsifier mixture led to increased daily weight gain in chickens (24). It is evident from the literature that LPL has the potential to enhance digestibility and energy levels in diets with reduced nutrient content.

Zhang et al. (2011) found that adding lysophosphatidylcholine to the diet of broiler chickens led to an increase in daily weight gain, regardless of the fat source during the initial period (25). This effect could be attributed

to the LPL's ability to enhance the emulsification of dietary lipids, forming small liposomes that are efficiently absorbed by the animals (26). The initial phase may have lacked lipase activity, while the weight gain in the final period could be linked to an increase in lipase activity, resulting in higher digestible fat levels through emulsification (11). Our study investigated the use of LPL supplements as a fat emulsifier in broiler chickens fed diets containing tallow and soybean oil for energy. Previous research suggested that young chickens have lower fat digestibility due to bile salt synthesis and recirculation compared to older birds (27). Including emulsifiers like LPL or bile salts in the diet generally enhances fat digestion and absorption in young birds, improving production performance (5). Therefore, it was anticipated that supplementing the diet with an appropriate emulsifier would enhance the efficiency of dietary fat utilization. However, our experiment did not observe any performance improvement in broiler chickens when LPL supplementation was used in young chickens, contrary to our initial hypotheses.

The impact of LPL supplementation on enhancing food conversion ratio did not show statistical significance in our research (P>0.05). Our findings were in line with Guerreiro et al. (2011), who found that the combined effect of fat and emulsifier did not have a significant impact on feed conversion ratio in broiler chickens throughout the entire study period (11). Sobotik et al. (2018) also supported our results by noting a significant difference in feed conversion efficiency in birds fed diets with varying levels of phosphatidic acid (5, 10, and 15 mg) up to 41 days of age (28). Similarly, Abbas et al. (2016) observed that emulsifier supplementation did not affect food conversion ratio in the early and overall breeding periods, but did have a significant impact in the final breeding period (23). While increasing fat content in the diet did not significantly influence food conversion ratio in the final and overall periods, there was an increase in food conversion ratio in the initial period with higher fat levels (23). Allahyari and his colleague (2017) found that the source of fat in the diet (soybean oil, soybean free fatty acids, and palm oil powder) did not significantly affect the average daily weight gain and feed conversion ratio of broiler chickens from 1 to 42 days (29).

In this study, the impact of LPL supplementation on feed consumption in broiler chickens was investigated. The treatment containing LPL supplement + 1.5% fat resulted in increased feed consumption compared to the treatment containing 1.5% of all types of fats without LPL supplement (P<0.05). Furthermore, it was observed that as the age of the birds increased, their energy requirements also increased, leading to an increase in feed consumption (P=0.04; data not presented in the table). It is suggested that LPL supplements may enhance the energy content and palatability of the feed, thereby influencing feed consumption positively. On the other hand, the lack of effect on feed consumption when using an emulsifier may be attributed to the low level of fat used in the diet, which did not significantly impact dust, palatability, and feed consumption. The study by Saleh et al. (2009) supports these findings, as they also reported no significant effect on feed consumption when using an emulsifier, possibly due to the low fat content in the diet (30). Additionally, they highlighted the positive effect of the supplement on saturated and unsaturated fatty acids at higher levels of dietary fat. Similarly, Zanganeh et al. (2018) found that LPL supplementation (1000 mg/kg) did not affect feed intake in broiler chickens (31). It is important to consider that the performance of broiler chickens may not be solely influenced by LPL supplementation, but rather by other factors such as the appropriate dosage of LPL in the diet, the energy level of the experimental diet, the experimental design, and the population size of the birds in each treatment. These factors may have compounded the effects and should be taken into account when interpreting the results.

3.2 Effects on blood metabolites

The findings regarding the impact of LPL supplementation, fat source, and diet level on the blood lipid composition of 14-day-old broiler chickens are presented in Table 4. Analysis of the combined effects revealed a significant influence of fat level and fat source on triglyceride levels in the blood serum of broiler chickens (P=0.04). Specifically, it was observed that in the absence of LPL supplementation, and a three percent fat level, the triglyceride content was lower compared to the three percent fat level with LPL supplementation, resulting in higher triglyceride levels (P<0.05). Furthermore, among the various interactions examined, only the impact of LPL supplementation and dietary fat source on LDL levels in the blood serum of 14-day-old broiler chickens was found to be significant. In particular, it was noted that in the absence of LPL supplementation but with safflower oil, there was a notable increase in serum LDL levels compared to other dietary arrangements (P<0.05). The addition of LPL to the diet led to a significant reduction in blood serum triglyceride levels in broiler chickens (P=0.03). Moreover, the utilization of different fat sources revealed that safflower oil

significantly elevated cholesterol and HDL levels in the blood serum of broiler chickens compared to beef tallow (P<0.04).

Table 4. The influence of lysophospholipid (LPL) on serum lipid indices in young chickens receiving different quantities and sources of fat-rich diets during starter phase.

Independent variable	Level or type	Triglyceride (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
LPL	0.025%	86.71[b]	156.46	76.34	63.29
	0	116.23[a]	162.34	73.03	61.75
	SEM	6.92	3.23	5.93	6.29
Fat source*	SO	88.71	173.69[a]	93.72[a]	66.23
	BT	90.85	146.14[b]	67.58[b]	57.39
	SO+BT	89.16	169.32[a]	84.97[ab]	60.91
	SEM	8.77	9.53	4.67	7.73
Fat level	1.5%	89.16	159.98	84.39	59.58
	3%	99.22	162.63	76.44	62.17
	SEM	9.36	7.32	5.42	5.92
Sex	Male	91.68	162.77	89.42	64.22
	Female	98.19	154.48	81.16	96.48
	SEM	9.24	9.37	4.82	8.81
P-value					
LPL (L)		**0.03**	0.43	0.74	0.38
Fat source (F_S)		0.36	**0.02**	**0.04**	0.69
Fat level (F_L)		0.33	0.24	0.23	0.37
Sex (S)		0.68	0.17	0.79	0.13
L×F_S		0.56	0.53	0.66	0.17
L×F_L		0.34	0.44	0.49	0.70
L×S		**0.05**	**0.03**	0.07	0.41
F_S×F_L		0.31	0.41	0.66	0.32
F_S×S		0.14	0.14	0.33	0.47
F_L×S		0.51	0.07	0.13	0.63
L×F_S×F_L		0.70	0.54	0.16	0.57
L×F_S×S		0.17	0.26	0.57	0.63
L×F_L×S		**0.04**	**0.04**	0.09	0.46
F_S×F_L×S		0.19	0.71	0.22	0.71
L×F_S×F_L×S		0.78	0.55	0.55	0.16

[a–b] Significantly different treatments in the starter phase are denoted by superscript letters with different subscripts at a significance level of P≤0.05

* SO = Safflower oil; BT = Beef tallow; SO+BF = 50% safflower oil+50% beef tallow

Jones et al. (1992) determined that an increase in dietary energy leads to elevated cholesterol production in peripheral cells of the body (32). High-density lipoprotein (HDL) facilitates the transfer of cholesterol back to the liver, where the enzyme lecithin-cholesterol acyltransferase acts by separating a fatty acid from lecithin and esterifying it with cholesterol to form a cholesterol ester that is soluble in HDL. HDL collects excess free cholesterol from peripheral cells and transports it to the liver for degradation (32). Consequently, with an elevated energy intake, the liver synthesizes more HDL to prevent a rise in blood serum cholesterol levels and facilitate their transfer to the liver for breakdown. Therefore, it can be inferred that the increase in cholesterol observed with the use of soybean oil has led to an upsurge in HDL production. The findings of these investigations suggest that LPL's impact on the serum lipid profile of broiler chickens may be more pronounced initially, resulting in reduced serum triglyceride levels. Jones et al. (1992) also suggested that the accelerated absorption and metabolism of ingested fat could explain the lower serum triglyceride levels in lecithin-fed pigs (32). One potential mechanism could be the faster clearance of chylomicrons from the bloodstream or a slower rate of their secretion into the blood. Further research is warranted to elucidate the precise mechanism by which emulsifiers affect blood serum compounds.

Contrary to the findings of Malapure et al. (2011), the concentration of total cholesterol in diets with varying levels

of LPL was observed to be lower compared to unsupplemented treatments after 42 days (33). Zhao et al. (2017) reported a decrease in LDL, total cholesterol, and triglycerides with LPL supplementation over a period of 14 days (34). In contrast to our own results, from 2010, Roy et al. demonstrated that glycerol polyethylene glycol ricinolate at concentrations of 1% and 2% led to reductions in LDL and total cholesterol levels (35). Fascina et al. (2009) found that serum total cholesterol levels decreased proportionally as the soybean oil fraction in the mixture increased (36). Conversely, same researchers reported that LDL and VLDL levels decreased linearly as dietary soybean oil levels increased, suggesting that sources of fat rich in unsaturated fatty acids can lower the average levels of these lipoproteins, thus potentially preventing arteriosclerosis in both humans and animals (36). These results align with the observations of Crespo and Steve-Garcia (2003), who noted a decrease in VLDL levels in birds fed sunflower or flaxseed oil compared to those fed tallow (37).

3.3 Effects on hepatic genes of lipid metabolism

Figures 1-5 present compelling data indicating that the inclusion of LPL, in conjunction with fat supplements, in experimental diets leads to significant changes in the expression of various hepatic genes ($P<0.05$). These genes are closely associated with the synthesis of fatty acids (*FASN*, *SCD*, and *ME1*), the synthesis of triglycerides (*GPAT* and *GPAM*), the oxidation of fatty acids (*CD36*, *CPT1A*, *CPT2*, and *ACOX1*), the transport of triglycerides (*apoB* and *apoVLDLII*), as well as the activity of transcription factors (*PPARα* and *PPARγ*). Furthermore, the statistical analysis conducted demonstrates that all the independent variables under investigation, along with specific interactions among them, exert a substantial impact on the expression levels of the selected genes in certain cases ($P<0.05$). This compelling evidence emphasizes the significance of LPL and fat supplements in modulating hepatic gene expression, thereby highlighting their potential therapeutic implications. In poultry, the liver is a key organ for lipid metabolism, and any disturbances in hepatic lipid metabolism can lead to conditions like fatty liver disease and excessive fat deposition (38). Transcriptome analysis has revealed that heat stress can alter gene expression related to nutrient metabolism in the liver of poultry, indicating the sensitivity of hepatic gene expression to environmental factors (39, 40). Additionally, microRNAs have been identified as important regulators of lipid metabolism in chicken liver, emphasizing the intricate regulatory network involved in hepatic lipid metabolism in poultry (41). As evidenced in prior scholarly investigations, although gene expression processes are inherently governed by genetics, the activation of these processes can be impacted by the intake of dietary nutrients (42, 43). Poultry research has revealed the significant influence of lysophospholipids on hepatic gene expression. By supplementing feed with lysophospholipids, hepatic lipid metabolism can be enhanced, leading to changes in the activity of lipid metabolizing enzymes and alterations in gene expression associated with lipid metabolism (44, 45). Furthermore, lysophospholipids have been observed to induce innate immune transdifferentiation of endothelial cells, resulting in prolonged endothelial activation through the upregulation of adhesion molecule gene expression and epigenetic reprogramming of endothelial cell activation (46). Additionally, lysophospholipids have been implicated in the regulation of hepatic lipid metabolism through microRNAs, such as miR-34a-5p, which can influence lipid metabolism in poultry liver (47).

In the evaluation of L2xx treatments as opposed to L1xx treatments illustrated in Figures 1-5, it was noted that LPL caused an elevation in the mRNA levels of *FASN*, *ME1*, *GPAT*, and *GPAM* in the hepatic samples obtained. Conversely, this emulsifier resulted in a reduction in the mRNA levels of genes associated with fatty acid oxidation (*CD36*, *CPT1A*, *CPT2*, and *ACOX1*) in the liver of the chickens under investigation. Notably, LPL did not produce a significant effect on hepatic *apoB*, *apoVLDLII*, *PPARs*, and *ME1*.

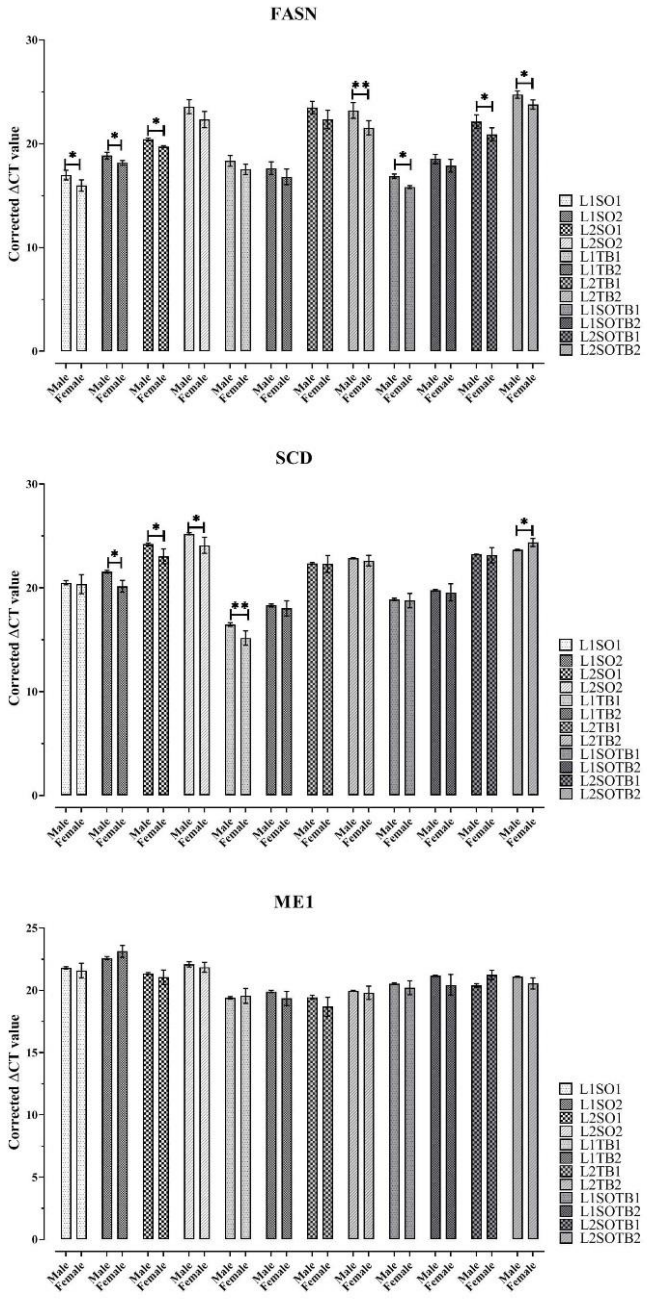

Figure 1. The impact of the experimental diets on the changes in CT values of fatty acid synthesis-related genes in the liver of broiler chickens. The diets included lysophosphate (L), safflower oil (SO), beef tallow (BT), and a combination of 50% safflower oil and 50% beef tallow (SOBT). The levels of lysophosphate and fat supplements were varied at 0% and 0.025% for L, and 1.5% and 3% for fat supplements. The results indicated that there were significant differences in the CT values of fatty acid synthase (FASN), malic enzyme 1 (ME1), and stearoyl-CoA desaturase (SCD) genes among the different diet groups. The significance levels were denoted by asterisks, with * representing differences significant at $P \leq 0.05$ and ** representing differences significant at $P \leq 0.01$. The error bars in the graph represent the standard error of the mean (SEM).

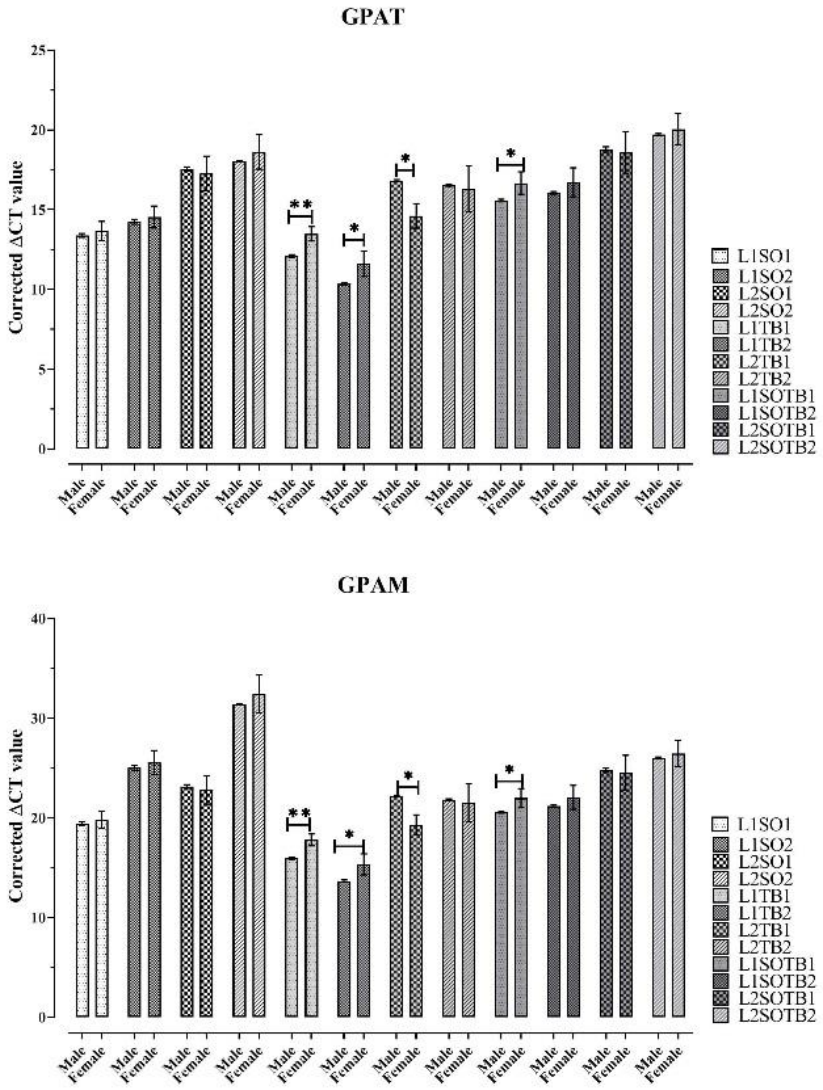

Figure 2. The effects of the experimental diets on the changes in CT values of genes related to triglycerids synthesis in the liver of broiler chickens. The diets used in the study consisted of lysophosphate (L), safflower oil (SO), beef tallow (BT), and a combination of 50% safflower oil and 50% beef tallow (SOBT). The levels of lysophosphate and fat supplements were varied at 0% and 0.025% for L, and 1.5% and 3% for fat supplements. Two specific genes, GPAT3 (glycerol-3-phosphate acyltransferase 3) and GPAM (glycerol-3-phosphate acyltransferase, mitochondrial), were analyzed in this study. The significance levels of the results were indicated by asterisks, with * representing differences that were statistically significant at a level of $P \leq 0.05$, and ** representing differences that were statistically significant at a level of $P \leq 0.01$. The error bars in the graph represented the standard error of the mean (SEM).

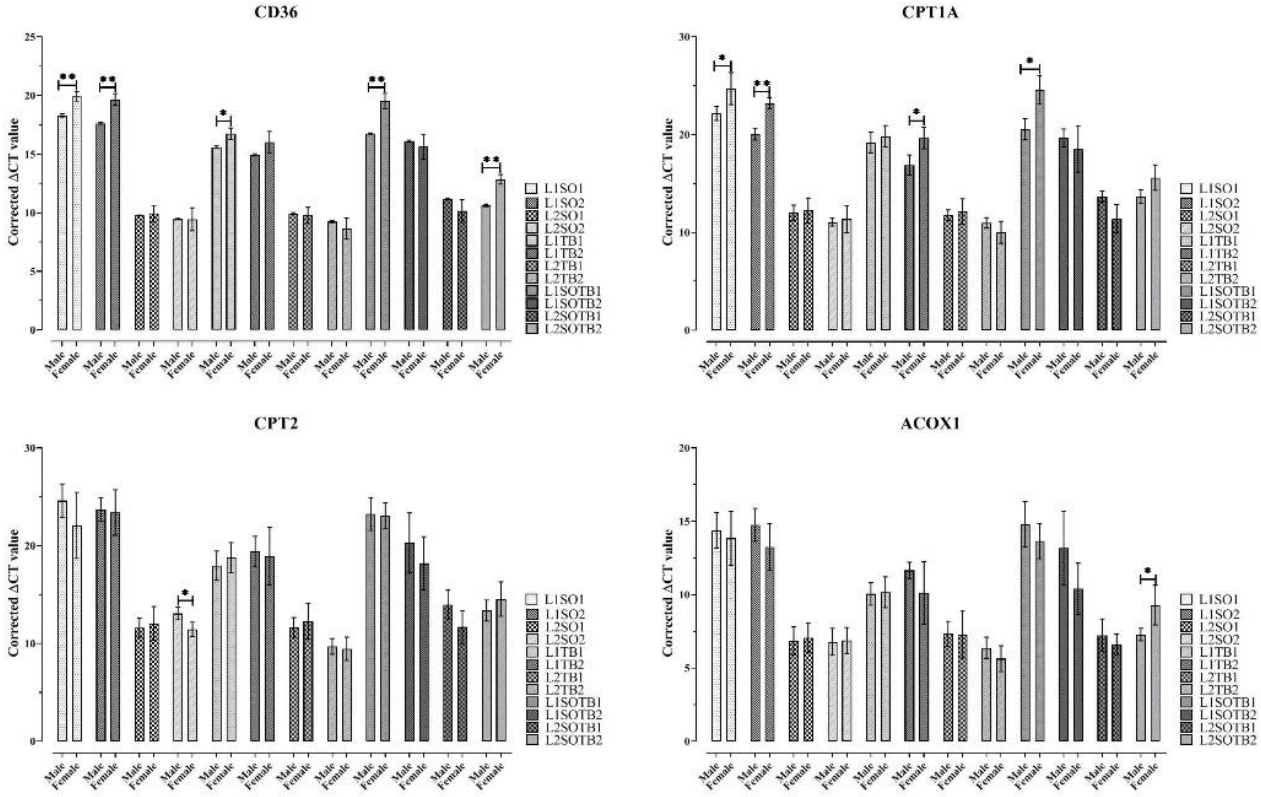

Figure 3. The effects of the experimental diets on alterations in CT values of genes related to fatty acid oxidation in the liver of broiler chickens. The diets tested included lysophosphate (L), safflower oil (SO), beef tallow (BT), and a combination of 50% safflower oil and 50% beef tallow (SOBT). The levels of lysophosphate and fat supplements varied at 0% and 0.025% for L, and 1.5% and 3% for fat supplements. The genes analyzed in the study were CD36 (CD36 molecule), CPT1A (carnitine palmitoyltransferase 1A), CPT2 (carnitine palmitoyltransferase 2), and ACOX1 (acyl-CoA oxidase 1). Statistical significance was indicated by asterisks, with * denoting significant differences at $P \leq 0.05$ and ** representing significant differences at $P \leq 0.01$. The error bars on the graph depict the standard error of the mean (SEM).

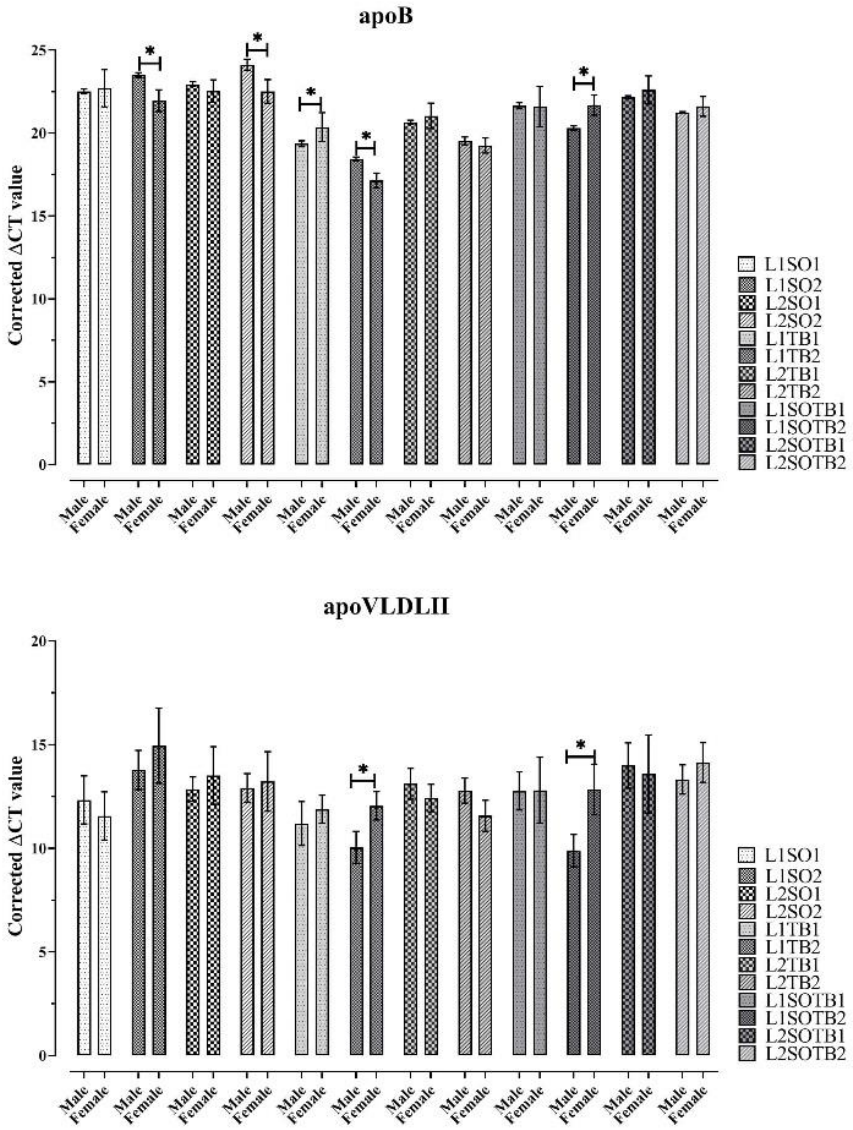

Figure 4. The impact of the experimental diets on changes in CT values of genes associated with triglyceride transport in the liver of broiler chickens. The diets examined were lysophosphate (L), safflower oil (SO), beef tallow (BT), and a combination of 50% safflower oil and 50% beef tallow (SOBT). The levels of lysophosphate and fat supplements were different, with L at 0% and 0.025%, and fat supplements at 1.5% and 3%. APOB stands for apolipoprotein B; apoVLDLII represents very low-density apolipoprotein II. Statistical significance was indicated by asterisks, with * representing significant differences at $P \leq 0.05$ and ** representing significant differences at $P \leq 0.01$. The error bars on the graph depict the standard error of the mean (SEM).

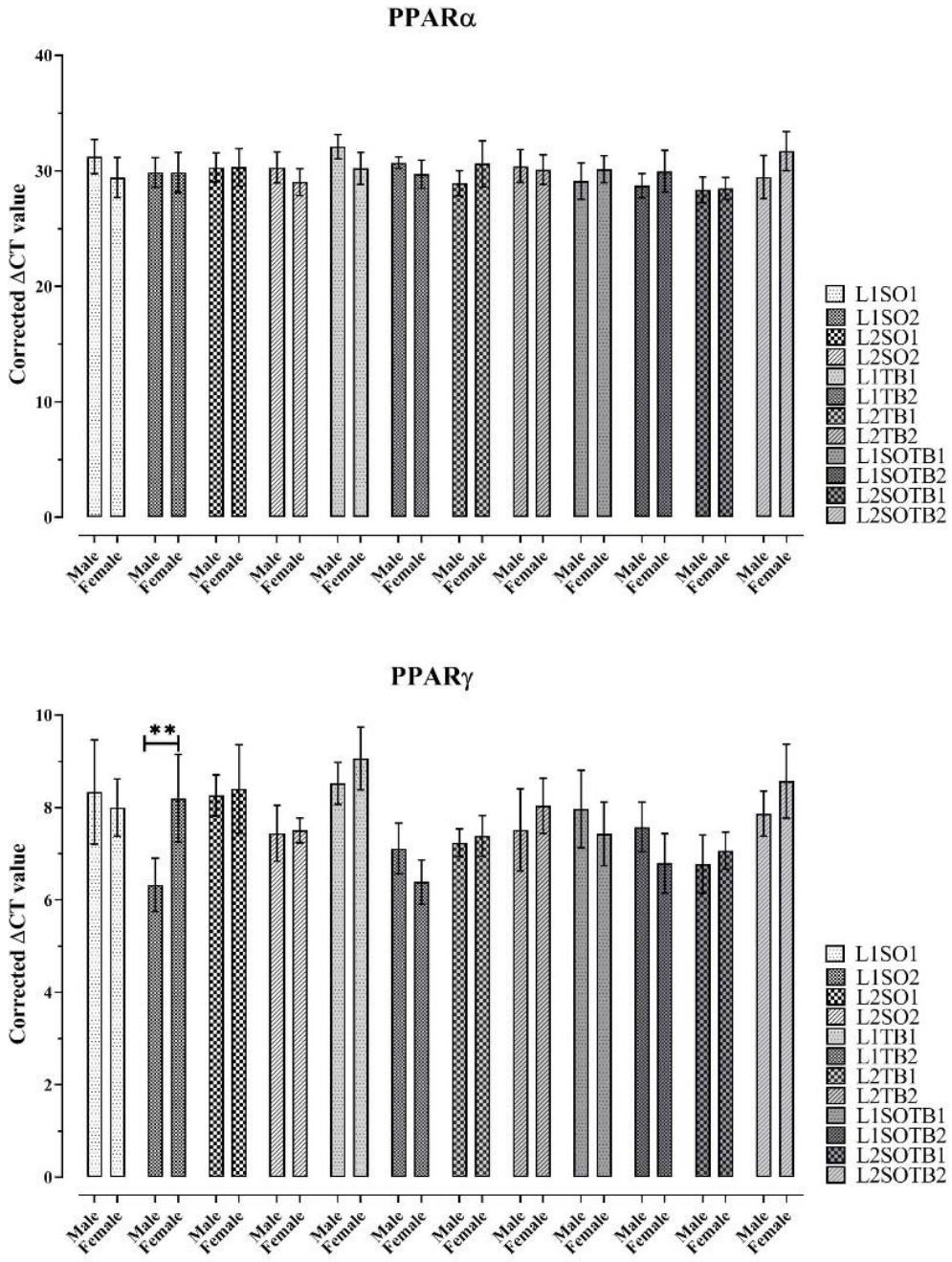

Figure 5. The effects of the experimental diets on the changes in CT values of transcription factor genes related to fatty acid synthesis in the liver of broiler chickens. The diets included lysophosphate (L), safflower oil (SO), beef tallow (BT), and a combination of 50% safflower oil and 50% beef tallow (SOBT). The levels of lysophosphate and fat supplements were varied at 0% and 0.025% for L, and 1.5% and 3% for fat supplements. The study also considered the involvement of peroxisome proliferator-activated receptor alpha (PPARα) and peroxisome proliferator-activated receptor gamma (PPARγ) in the observed changes. The significance levels of the results were indicated by asterisks, with * representing differences that were statistically significant at a p-value of ≤ 0.05, and ** representing differences that were statistically significant at a p-value of ≤ 0.01. The error bars in the graph represented the standard error of the mean (SEM).

Furthermore, lysophospholipids have been associated with the suppression of hepatic fatty acid oxidation, which can lead to increased VLDL synthesis and contribute to diet-induced obesity and metabolic disorders (48, 49). The results presented in Figure 3 support the previous studies indicating that emulsifiers like lysophospholipids inhibit the fatty acid oxidation process in the liver. This inhibition of fatty acid oxidation influences lipid and glucose homeostasis by reducing mitochondrial fatty acid oxidation in the liver (50). Furthermore, the reduction in hepatic fatty acid oxidation due to lysophospholipid absorption increases the availability of fatty acid substrates for triglyceride biosynthesis (48). The increased mRNA levels of *GPAT* or *GPAM* detected in our investigation may be linked to elevated fatty acid concentrations within hepatocytes, potentially stemming from the inhibitory impact of LPL on the fatty acid oxidation pathway. The dysregulation of lysophospholipid metabolism has been linked to conditions like obesity, fatty liver disease, and metabolic syndrome (50, 51). Moreover, lysophospholipids have been shown to impact lipid absorption in the small intestine, highlighting their role in lipid digestion and metabolism (52).

The utilization of methylmalonate for the synthesis of branched-chain fatty acids in chicken liver has been studied, showing that in the absence of malonyl-CoA, methylmalonyl-CoA is utilized for fatty acid synthesis at a slow rate (53). Additionally, dietary components like soybean phospholipids have been found to depress hepatic fatty acid synthesis, potentially decreasing malonyl-CoA concentration and enhancing fatty acid oxidation rates (54). Moreover, the up-regulation of lipogenesis activity in the liver of broiler chickens raised under heat stress conditions indicates abnormal fat synthesis in the liver (39). This abnormal fat synthesis can have implications for the fatty acid composition of poultry meat, as the presence of fatty acids in the diet and their synthesis in the liver influence the fatty acid profile of poultry muscle (55).

To summarize, based on our research that supports existing literature, lysophospholipids have a significant influence on regulating fatty acid metabolism in the liver of poultry. This impact extends to crucial processes like fatty acid oxidation and triglyceride biosynthesis. Gaining a comprehensive understanding of how lysophospholipids and various dietary and environmental factors affect fatty acid synthesis in poultry liver is vital for enhancing poultry nutrition and ensuring high-quality meat production.

In our study, the hepatic genes associated with the synthesis of fatty acids were repressed by the utilization of beef tallow ($P<0.05$). Studies have shown that the composition of dietary fat can significantly impact the transcriptional profile of pathways associated with lipid metabolism in the liver (56). The chemical composition of dietary fat sources can influence the expression of genes involved in lipid metabolism in poultry, affecting fat deposition and composition (57). The type of dietary lipid sources can influence fatty acid composition, enzyme activities, and gene expression related to lipid metabolism in poultry, showcasing the importance of lipid sources in poultry diets (58). The observed disparity in the regulatory impact of beef tallow (BT) and safflower oil (SO) on specific genes could potentially be attributed to the contrasting fatty acid compositions of these two substances, as supported by corroborating evidence.

Several studies have provided evidence for sex-specific disparities in the expression of hepatic genes involved in drug metabolism and steroid metabolism (59). These disparities are influenced by the action of growth hormones on individual genes, such as P450s, as well as other sex-specific liver genes at the transcriptional level. Furthermore, investigations conducted on pigs and chickens have utilized transcriptome analysis of liver samples to identify genes associated with lipid metabolism, thereby shedding light on the intricate relationship between liver metabolism and lipid-related phenotypes (60). In our study, the observed impact of sex on lipid gene regulation in the liver could potentially be attributed to differences in basic metabolism and hormonal variations, which warrant further meticulous exploration.

The choice of fat sources in poultry diets plays a critical role in the regulation of lipid metabolism-associated genes within the liver. Variations in dietary fats have the potential to influence gene expression, enzyme functions, and lipid storage in poultry, underscoring the importance of customized dietary approaches to enhance lipid metabolism in poultry farming.

3.4 Effects on microbial population of the cecum

The outcomes of LPL supplementation, the type of fat source, and the fat level in the diet on the microbial population of the cecum of broiler chickens at 14 days of age are presented in Table 5. Interactions, whether triple or double, did not yield a significant impact on the population of *Lactobacillus* and *Escherichia coli* in the cecum of broiler chickens ($P>0.05$). LPL supplementation notably boosted the population of *Lactobacillus* bacteria in the cecum of

broiler chickens (P=0.02). Furthermore, the addition of three percent fat to the vegetable diet significantly decreased the population of *Escherichia coli* bacteria (P=0.03). Polycarpo et al. (2016) did not detect any growth in total *Enterobacteriaceae* in rhizome samples when LPL was administered at 14 days (19). They also noted that there was no interaction effect between lipid sources (soybean oil and calf tallow) and LPL supplementation (zero and one kg/ton) on Gram-positive coccidia. Nevertheless, the presence of LPL reduced the number of Gram-positive cocci in the jejunum of broiler chickens fed with a tallow-containing diet. Additionally, LPL feeding resulted in similar outcomes to the soybean oil diet, with lower levels of total anaerobic microorganisms. The bacterial count in jejunum samples indicates that LPL feeding is effective in diets containing tallow, mitigating the adverse effects of high saturated fatty acid content on the number of Gram-positive cocci. This reduction can be attributed to the antimicrobial properties of LPL, which may manifest in two distinct ways (19).

Table 5. The influence of lysophospholipid (LPL) on the cecum microbial population of young chickens receiving different quantities and sources of fat-rich diets during starter phase.

Independent variable	Level or type	*Lactobacillus spp.* (Log10cfu/g)	*E-coli* (Log10cfu/g)
LPL	0.025%	8.21[a]	4.16
	0	7.88[b]	4.35
	SEM	0.17	0.09
Fat source*	SO	7.33	4.33
	BT	7.56	4.62
	SO+BT	7.49	4.51
	SEM	0.21	0.15
Fat level	1.5%	7.73	4.71[a]
	3%	7.78	4.09[b]
	SEM	0.09	0.19
Sex	Male	7.66	4.42
	Female	7.70	4.55
	SEM	0.16	0.21
P-value			
LPL (L)		**0.02**	0.57
Fat source (F_S)		0.22	0.72
Fat level (F_L)		0.67	**0.04**
Sex (S)		0.71	0.53
L×F_S		0.38	0.19
L×F_L		**0.03**	**0.05**
L×S		**0.05**	0.21
F_S×F_L		0.71	0.78
F_S×S		0.34	**0.03**
F_L×S		0.39	0.61
L×F_S×F_L		0.61	0.47
L×F_S×S		0.52	0.26
L×F_L×S		**0.04**	**0.05**
F_S×F_L×S		0.24	0.65
L×F_S×F_L×S		0.31	0.66

[a–b] Significantly different treatments in the starter phase are denoted by superscript letters with different subscripts at a significance level of $P \leq 0.05$

* SO = Safflower oil; BT = Beef tallow; SO+BF = 50% safflower oil+50% beef tallow

The direct action of LPL on Gram-positive microorganisms involves altering the permeability of their cell membranes, resulting in damage to the bacterial membrane integrity due to ion imbalance (61). Another reason for its effectiveness is the higher utilization of tallow, which leads to a reduction in substrate availability in the intestinal lumen. These research findings suggest that LPL can effectively reduce the production of growth-reducing metabolites by Gram-positive bacteria, which is a beneficial factor associated with antibiotic use (62). Numerous studies have demonstrated the impact of LPL on the intestinal microbiota of broiler chickens, particularly on specific microorganisms such as *Enterococcus faecium* and

Clostridium perfringens, which play a role in lipid digestion (63).

4 Conclusion

The addition of LPL has been demonstrated to significantly boost the population of lactobacillus and reduce blood serum triglyceride levels in broiler chickens. Nevertheless, it is important to highlight that the incorporation of LPL did not yield any significant impacts on performance or other variables under investigation. Consequently, it is not possible to definitively assert that the utilization of this supplement will directly influence the performance of broiler chickens within the 1 to 14-day timeframe in the context of this specific experiment. Moreover, lysophospholipids play a substantial role in hepatic gene expression and lipid metabolism in poultry, affecting crucial processes like lipid synthesis, absorption, and metabolism. Understanding the function of lysophospholipids in poultry liver function is crucial for enhancing poultry nutrition and health. Notably, gender also plays a significant role in lipid metabolism-related genes in poultry liver, with research emphasizing the importance of comprehending sex-specific variations in gene expression and the potential for interventions to regulate lipid metabolism pathways. The outcomes of the present study should be further explored with different genes and fat sources. Nevertheless, these initial findings could be viewed as a preliminary step towards uncovering the precise pathway to achieving efficient poultry production to fulfill economic requirements for farmers and reducing consumer health concerns by ensuring higher quality outputs.

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Conflict of Interest

The authors declare no conflicts of interest with any organization or entity. Furthermore, they have no personal or professional relationships, affiliations, knowledge, or beliefs related to the subject or materials discussed in this paper.

Author Contributions

A .A. was responsible for formulating the project, developing the key conceptual ideas, outlining the proof, and handling most of the technical aspects. M. Z. conducted the numerical calculations for the proposed experiment and independently validated the trial's numerical outcomes. Through collaborative discussions, A. A. authored the manuscript, while M.Z. meticulously reviewed and edited it.

Data Availability Statement

Data are available from the corresponding author upon reasonable request.

Ethical Considerations

The Animal Care and Animal Handling rules and regulations, approved by the Animal Ethics Committee of Yasouj University, were strictly followed for all live-animal-related procedures. Throughout the experiment, animals were consistently supervised by veterinary professionals, ensuring that all handling standards were adhered. The authors of this report affirm that the data presented herein are solely the result of their own research. It is important to note that there was no conflict of interest that could have influenced the results in a biased manner, favoring any individual or entity.

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