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Physical Form of Diet Influence the Liver Function, Blood Biochemistry, and External Body Measurements in Broiler Chickens Exposed to Carbon Tetrachloride Toxicity

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Abstract

This 2 × 3 factorial experiment aimed to evaluate the single and interactive effects of feed form (pelleted, crumble and mash) and carbon tetrachloride (CCl_4) intoxication on liver function, blood parameters, and certain external body dimensions in broiler chicken up to day 42 of age. The six experimental treatments were examined with a completely randomized block design in six replicates of 13 birds each using 468 10-day old female Arbor Acres (320±10g) chicks. For CCl_4 intoxication, birds were intraperitoneally injected with 0.5, 0.5, and 0.75 mL/kg body weight CCl_4 in olive oil at a ratio of 1: 1, v/v in days 14, 21, and 28 of age. Results indicated that feeding the pelleted diet increased daily weight gain (DWG), liver fat percentage (LFP), and improved feed conversion ratio compared with those received crumble and mash diets *($P < 0.05$)*. The broilers receiving the pelleted diet had a greater breast angle (BrG) than those feeding with the mash diet *($P < 0.05$)*. Exposure to CCl_4 decreased breast width (BrW), breast girth (BrG), and BrA of the treated birds *($P < 0.01$)*. The best multivariate linear model for prediction of liver fat percentage achieved by the Forward modeling approach in SAS involving serum TC, LDL TP, TBIL, LDH, BrA, and ShL with R^2=0.3011. It was concluded that feeding diets in pelleted form may cause a greater performance loss and liver dysfunction when broiler chickens are fed with contaminated feed resources. Moreover, prediction of LFP using multivariate linear models based on blood constituents and external body measurements could not be convincing as for no model R^2 exceeds 0.31 likely due to the lack of strong correlation between LFP and the considered predictors.

Introduction

With the expansion of the commercial broiler chicken industry, metabolic disorders have become emerging serious hazards in bird's health and profitability (Savard *et al.,* 2013). Fatty liver, among many other metabolic syndromes, has not received adequate attention in broiler research while it plays a significant role in the pathogenesis of many liver and kidney diseases and may directly cause low performance and a great economic loss (Zhang *et al.,* 2018). It is well known that excessive lipid accumulation in the liver is promoted by enhanced mobilization of fatty acids from fat depots, *de novo* synthesis of fatty acids, dietary triglycerides, and a ceased fatty acid oxidation due to unfair influx vs. removal of lipids (Hong *et al.,* 2019). Such biochemical facts are almost all anticipated in broiler flocks where birds are virtually raised with free access to high energy diets in confined circumstances without adequate physical activity. More complications arise when birds are maintained on diets contaminated with mycotoxins or other environmental contaminants (Murugesan *et al.,* 2015; Mokubedi *et al.,* 2019).

Carbon tetrachloride as an environmental toxicant has frequently been used as a promising method by several *in vitro* and *in vivo* experiments to study animal toxicity (Manibusan *et al.,* 2007; Beheshti Moghaddam *et al*., 2016; Vahed *et al*., 2016). Toxicity of CCl_4 realizes through its biotransformation by the cytochrome P_{450} in the endoplasmic reticulum of the hepatocytes to produce free radicals dominated by trichloromethyl (CCl_3•) (Zhang *et al.,* 2018). Moreover, Baradaran *et al*. (2019) reported remarkable down-regulation in the expression of CAT, GPx, and Mn-SOD hepatic genes in CCl_4-challenged broiler chicks.

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Unfortunately, birds suffering fatty liver, irrespective of the relevant risk factors and etiology, are not diagnosed before clinical symptoms appear. Therefore, management of the promoting environmental and nutritional factors such as feed particle size (Shirani *et al*., 2018), feed physical form (Mohammadi Ghasem Abadi, *et al*. 2019), as well as contamination of diet with toxicants (Baradaran *et al*., 2019), must receive priority for the prevention of fatty liver in broiler farms. With the short lifetime of broiler chicken, appropriate non- invasive indications for diagnosis of birds suffering from the fatty liver are welcomed in broiler research and veterinary practice. A liver fat content beyond five percent is considered as fatty liver (Bedogni *et al.,* 2014; Nicolas, 2019). However, access to perfect data on liver fat using noninvasive methods is not quite incredible but by autopsy of the expired birds. In the current study, we tried to explore the association between many external body dimensions as well as blood biochemical parameters with liver fat percentage hopeful to find a multivariate linear model to predict liver fat percentage in liver broiler chickens. Therefore, this study intended to investigate the effects of feed form on liver function, blood constituents, and certain external body measurements in broiler chickens exposed to CCl_4 toxicity and to provide multivariate linear models for prediction of liver fat percentage using the evaluated parameters.

Materials and methods

Animals and diets

Four hundred sixty-eight 10-day old female Arbor Acres broiler chicks at the highest proximity of size were chosen and used to examine the effects of six treatments in a 2 × 3 factorial arrangement with a completely randomized block design in six replicates of 13 birds each. The birds were selected from a flock consisting of 2000 straight run chicks which provided from a local hatchery and housed in a power-ventilated grow-out house where raised in a floor pen up to day 10 of age. During this early period, birds were grown on a pelleted starter diet (Table 1) and water *ad libitum* under a 23:1 light to darkness lightening regimen. All procedures carried out in this experiment were reviewed and approved by the Animal Care and Use Committee of Lorestan University, Khorramabad, Iran. The ambient temperature and relative humidity were kept at 32±1°C and 60±5%, respectively.

At the initiation of day 11, the selected birds were distributed into 36-floor pens (0.9 × 1.8 m) where they spent three days for acclimatization and then subjected to the experimental treatments up to day 42 of age. Treatments consisted of a grower diet (Table 1) presented in three physical forms (mash, crumble, and pelleted) and fed to birds with or without CCl_4 injection. For CCl_4 intoxication, birds were intraperitoneally injected with 0.5, 0.5, and 0.75 mL/kg body weight CCl_4 in olive oil at a ratio of 1: 1, v/v in days 14, 21, and 28 of age, respectively. The pelleting process was accomplished at a temperature of 90°C. The primary mash diet was pelleted and then pellets were crumbled in a roller mill, resulting in a crumbled diet. During the experimentation period, days 11 to 42, the chicks received a grower diet and water *ad-libitum* (Table 1).

Table 1. Ingredients and nutrient composition of diets.

Ingredients (%)	Starter (1-10 day)	Grower (11-42 day)
Yellow maize	58.34	63.08
Soybean meal (44% CP)	36.38	29.36
Soybean oil	1.50	4.10
Calcium phosphate	1.24	1.40
CaCO3	1.34	1.20
DL-Methionine	0.28	0.18
L-Lysine HCL	0.28	0.04
Salt	0.14	0.14
Mineral Premix[1]	0.25	0.25
Vitamin Premix [2]	0.25	0.25
Nutrient composition		
ME (Kcal/kg)	3000	3176
Crude protein (%)	21.50	17.00
Lysine (%)	1.44	1.00
Potassium (%)	0.80	0.76
Methionine (%)	0.56	0.50
Methionine + Cystine (%)	1.08	0.55
L-Threonine (%)	0.97	0.72
Calcium (%)	096	0.80
Available P (%)	0.48	0.41
Na (%)	0.20	0.20
Cholorine (%)	0.20	0.20

[1] Each kilogram contains: 120.0 mg manganese; 110.0 mg Zinc; 16.0 mg copper; 300.0 mg selenium; 1250 mg iodine; 20.0 mg iron.

[2] Each Kilogram contains: Vitamin A, 12000 IU; vitamin D_3; 5000 IU; vitamin E 80 IU; vitamin K_3; 3.2 mg; vitamin B_1; 3.2 mg; vitamin B_2; 8.6 mg ; vitamin B_3; 20 mg; vitamin B_5; 65 mg; vitamin B_6; 4.3 mg; vitamin B_9; 2.2 mg; vitamin B_{12}; 0.017 mg; vitamin H_2; 0.30 mg; choline chloride; 1700, mg.

Performance data

Live body weight (BW) and feed intake (FI) were recorded in days 14 and 42 of age and data were utilized to generate daily weight gain (DWG), average daily feed intake (DFI), and feed conversion ratio (FCR). Mortality was recorded through the experiment upon occurrence. The European production efficiency index (EPEI) was calculated based on the method provided by Euribrid (1994).

EPEI (%) = (Body weight (Kg) × livability (%)) / (Age (days) × feed conversion ratio) ×100

Blood constituents

At the end of the experiment (Day 42), all the birds were weighed, killed by puncturing the jaguar veins and carotid arteries, scalded, de-feathered mechanically, and eviscerated manually. Individual samples of whole uncoagulated blood were collected and centrifuged at 1800 ×*g* for 15 min. The collected sera preserved at -20°C pending analysis. Concentrations of serum glucose (GLU), albumin (ALB), triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), total protein (TP), total bilirubin (TBIL), direct bilirubin (DBIL), and the activity of serum alkaline phosphatase (ALP), aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were determined using an auto-analyzer machine (Clima Ral. Co, Barcelona, Spain). The analyzer employed enzymatic procedures using SEPPIM Diagnostic Kits (SEPPIM S.A.S., Sees, France) based on the method reported by Elliott (1984).

Liver fat and liver health scoring

Livers from all the slaughtered birds were weighed and then macroscopically appraised for color and apparent health. Liver scores on a 4-point scale were assigned as follows: the most severe lesion and color alteration was given score 3, where normal = 0, as described by Trott *et al.* (2014) with a slight modification. After scoring, the liver samples kept at -4°C pending fat extraction based on the method devised by Folch *et al.* (1957). Briefly, about 1 g from each liver tissue weighed, added to chloroform/methanol (2/1) to a final volume of 20-times the volume of the tissue sample vortexed for one minute and allowed to stand with agitation for 2 h. The separated liquid was filtered through Whatman #1 filter paper into a 100-mL 54 graduated cylinder, and 5 mL of 7.3% Potassium chloride solution was added and blended. After phase separation, the top layer was carefully drained off. Total lipids are determined gravimetrically after evaporating the solvent. The samples were then dried, weighed, and total lipid weight was expressed as the percentage of liver fat against the total liver weight.

External body dimensions

Shank length (ShL), shank width (ShW), breast length (BrL), and breast width (BrW) were recorded using a digital caliper at the accuracy of 0.01 mm. Breast girth (BrG) was measured using a tape measure at the most plumpy and fleshy point at the accuracy of 0.5 cm. The breast angle (BrA) was determined using a breast angle meter at the accuracy of 1 degree. All measurements were taken at day 42 of age according to the procedure adopted by Khosravinia *et al.* (2006).

Fear test data

The tonic immobility reaction (fear test) was induced in all birds during day 40 of age based on the method described by Ghareeb *et al.* (2014). Briefly, an individual bird was placed on its back and restrained with one hand on its sternum for 45 seconds while its head and neck were kept with the other hand. Towards the end of the holding period, hand pressure was slowly lifted and the duration of tonic immobility reaction, as the time needed by the bird until righting and standing was recorded using a stopwatch.

Statistical analysis

Performance and blood-related data were subjected to a two-way analysis of variance using the GLM procedures of SAS 9.1 (SAS Institute, 2003). The following statistical model has used the effects of feed presentation form (mash, crumble, and pellet), CCl_4 injection, and their interactions.

$$Y_{ijkl}=\mu+FF_i+TC_j+(FF\times TC)_{ij}+B_k+\varepsilon_{ijkl}$$

where Y_{ijkl} is the variance associated with parameter; μ is the overall mean; FF_i is the feed physical form effect; TC_j is the CCl_4 administration effect; $(FF\times TC)_{ij}$ is the interaction effect; and e_{ijkl} is the experimental error effect. Multiple treatment comparisons were performed using the Tukey test (Kramer, 1956). The scores assigned to each bird for apparent liver health were statistically analyzed using PROC FERQ in the same statistical analysis software (SAS Institute, 2003) for their frequency. For all tests, the maximum likelihood for type-I error was considered at 5% *($P < 0.05$)*. Logistic regression analyses were undertaken to identify the best combination of parameters for predicting liver fat percentage using the forward, backward, and stepwise approaches in SAS modeling options. Forward and Backward elimination option with a threshold of P= 0.05 was adopted to pick up the covariates for the final model. We incorporated 17 predictor variables in each binary logistic regression analysis model. The body measurements including ShL, ShW, BrL, BrW, BrG, and BrA as well as a fear test (FT) and blood parameters including TG, TC, LDL, TP, TBIL, ALT, AST, LDH, ALB, TBIL, and DBIL were analyzed using multiple linear regression techniques (Draper and Smith, 1981).

Results

Mean DWG in the broilers fed on the pelleted diet increased by 7.03 g (19.08%) and 3.38 g (9.17%) compared with those received crumble and mash diets, respectively ($P < 0.05$; Table 2). Mean FCR and EPEI also improved in the pelleted diet-receiving birds than those offered to crumble diet in days 14 to 42 age ($P < 0.05$; Table 2). Broilers feeding with the mash diet showed a greater FI than those maintained on the crumble diet in days 14 to 42 of age. Mean weight gain was reduced in the birds fed on the crumble diet and exposed to CCl_4 toxicity at day 42 of age *($P < 0.05$)*.

Table 2. Performance parameters in broiler chickens challenged with CCl_4 and fed with a diet provided in three pelleted, crumble and mash form at day 14 - 42 of age.

Diet form	CCl_4[1] (mL/kg)	Daily weight gain (g)	Daily feed intake (g)	Feed conversion ratio	European Production Efficiency index
Pelleted		36.83^{a}	47.90^{ab}	1.32^{b}	286.33^{a}
Crumble		29.80^{b}	44.75^{b}	1.42^{a}	197.03^{b}
Mash		33.45^{ab}	48.65^{a}	1.48^{ab}	233.48^{ab}
SEM		1.171	1.144	0.781	17.49
	0	33.18	46.45	1.46	239.43
	1.75	33.53	46.75	1.41	238.46
	SEM	0.956	0.934	0.045	14.28
Pelleted	0	36.54^{ab}	48.44	1.38^{bc}	253.93^{ab}
Pelleted	1.75	39.11^{a}	47.36	1.25^{c}	318.74^{a}
Crumble	0	29.73^{c}	45.30	1.53^{ab}	195.85^{b}
Crumble	1.75	29.86^{c}	44.21	1.52^{ab}	198.21^{b}
Mash	0	35.27^{ab}	46.70	1.33^{bc}	268.52^{ab}
Mash	1.75	31.63^{bc}	50.61	1.62^{a}	198.44^{b}
SEM		1.657	1.619	0.078	24.74
P-value					
Diet		0.0011	0.0548	0.0383	0.0050
CCl_4		0.7970	0.3355	0.4764	0.9622
Diet × CCl_4		0.0542	0.3179	0.0333	0.0385

[a,b,c] Means with different superscript within a column differ significantly ($P < 0.05$). [1]CCl_4 has intraperitoneally injected a dose of 0.5, 0.5, and 0.75 mL/kg body weight diluted in olive oil at a ratio of 1: 1 (v/v) at days 14, 21, and 28 of age, respectively.

Table 3. Mean serum concentration of serum lipids fractions in broiler chickens challenged with CCl_4 and fed with a diet provided in three pelleted, crumble and mash form at day 42 of age.

Diet form	CCl_4[1] (mL/kg)	Triglyceride (mg/dL)	Total cholesterol (mg/dL)	Low density lipoprotein-cholesterol (mg/dL)	Liver fat percentage
Pelleted		131.52	181.65	49.09	6.60^{a}
Crumble		135.10	169.88	44.26	5.59^{ab}
Mash		149.21	170.31	47.95	5.08^{b}
SEM		9.360	7.98	2.95	0.186
	0	135.27	168.77	49.20	5.37^{b}
	1.75	141.96	179.05	44.99	6.14^{a}
	SEM	7.46	5.95	2.26	0.148
Pelleted	0	115.64	163.81^{c}	41.91^{c}	6.38^{a}
Pelleted	1.75	147.40	199.66^{a}	56.27^{a}	6.82^{a}
Crumble	0	143.72	173.49^{bc}	45.90^{abc}	5.20^{b}
Crumble	1.75	126.48	166.84^{b}	42.62^{c}	5.98^{ab}
Mash	0	146.44	168.29^{bc}	47.18^{b}	4.54^{c}
Mash	1.75	151.98	172.64^{bc}	48.72^{abc}	5.63^{ab}
SEM		12.71	11.08	3.94	0.275
P-value					
Diet		0.4032	0.6752	0.4429	<.0001
CCl_4		0.5339	0.1128	0.2028	0.0005
Diet × CCl_4		0.1430	0.0020	0.0412	0.04747

[a,b,c] Means with different superscript within a column differ significantly ($P < 0.05$). [1] CCl_4 was intraperitoneally injected at a dose of 0.5, 0.5, and 0.75 mL/kg body weight diluted in olive oil at a ratio of 1: 1 (v/v) at days 14, 21, and 28 of age, respectively.

The serum concentration of TC and LDL-c influenced by diet physical form × CCl_4 interaction *($P < 0.05$)*. The birds fed on the pelleted diet and received CCl_4 injection exhibited an increased TC

and LDL-c serum concentration compared with those grown on the crumble diet and exposed to CCl_4 toxicity *($P < 0.05$)*. The liver fat percentage in the birds receiving the pelleted diet was greater than those fed on the mash diet *($P < 0.05$)*. CCl_4 injection caused a significant increase in LFP compared with those that did not exposed the same challenge *($P < 0.05$)*. Feeding with the pelleted diet concomitant with CCl_4 administration caused a greater liver fat deposition compared to the other treatment combinations ($P < 0.05$; Table 3).

Serum activity of ALP, AST, ALT, and LDH was not modified by feed physical form and CCl_4 injection at day 42 of age ($P > 0.05$; Table 4). Serum AST activity, however, elevated in the CCl_4-injected birds when they fed with the crumble and pelleted diets ($P < 0.05$; Table 4).

Table 4. Mean serum activity of enzymes in blood broiler chickens challenged- CCl_4 fed with pellet, crumble, and mash form diet at day 42 of age.

Diet form	CCl_4[1] (mL/kg)	Alkaline phosphatase (U/L)	Aspartate aminotransferase (Ul/L)	Alanine aminotransferase (Ul/L)	Lactate Dehydrogenase (Ul/L)
Pelleted		2037.70	5.36	256.90	3142.50
Crumble		2006.25	5.30	260.41	3079.74
Mash		1999.43	4.85	265.22	3174.80
SEM		25.144	0.372	5.321	80.926
	0	2000.81	4.52	259.99	3145.68
	1.75	2028.12	5.83	262.37	3118.13
	SEM	33.91	0.295	4.22	61.683
Pelleted	0	2000.52	5.06[ab]	252.48	3097.56
Pelleted	1.75	2074.89	5.68[a]	263.32	3187.34
Crumble	0	2000.89	5.01[ab]	252.50	3097.27
Crumble	1.75	2011.60	5.67[a]	269.31	3063.87
Mash	0	2001.00	4.07[b]	266.97	3243.81
Mash	1.75	1997.87	4.54[ab]	270.46	3105.33
SEM		34.46	0.540	7.255	113.60
P-value					
Diet		0.4849	0.2852	0.3590	0.2716
CCl_4		0.3436	0.0237	0.0884	0.7579
Diet × CCl_4		0.4875	0.0087	0.6829	0.6576

[a,b] Means with different superscript within a column differ significantly ($P < 0.05$). [1] CCl_4 has intraperitoneally injected a dose of 0.5, 0.5, and 0.75 mL/kg body weight diluted in olive oil at a ratio of 1: 1 (v/v) at days 14, 21, and 28 of age, respectively.

No significant differences were observed in serum concentrations of GLU, TP, TBIL, DBIL, and ALB in the birds subjected to the experimental treatments at day 42 of age ($P > 0.05$; Table 5). The serum concentration of GLU, however, increased in the broilers maintained on the pelleted diet and injected with CCl_4 and in those fed on the mash diet without CCl_4 injection compared with those receiving the pelleted diet without CCl_4 administration ($P < 0.05$; Table 5). The serum concentration of TP was greater in the birds receiving the pelleted diet and injected with CCl_4 ($P < 0.05$; Table 5). The birds which fed on the pelleted and mash diets with CCl_4 injection showed greater serum TBIL concentration than those receiving the pelleted diet without exposure to CCl_4 toxicity ($P < 0.05$; Table 5). The birds are grown on the pelleted diet and challenged with CCl_4 also demonstrated a greater concentration of DBIL than other broilers ($P > 0.05$; Table 5).

Apparent liver health, scored using a 4-grade scale, was affected by the physical form of the diet, CCl_4 injection as well as their interrelations effects ($P < 0.05$; Table 6). For score 0, indicating a healthy liver, the relative frequency was greater (38.84%) in the birds maintained on the mash diet. The broilers receiving pelleted diet showed higher frequency for score 2 and 3 on day 42.

Mean BrG was greater in the broilers receiving the pelleted diet than those fed with the mash diet ($P < 0.05$; Table 7). Exposure to CCl_4 toxicity resulted in a significant decrease in BrW, BrG, and BrA *($P < 0.05$)*. Mean ShW, BrW, BrG, and BrA were altered by feed presentation form × CCl_4 interaction, whereas ShW was greater in the broilers feeding with a crumble diet and exposed to CCl_4 toxicity and BrW increased in broilers receiving the pelleted and mash diets without CCl_4 exposure than those feeding with the mash diet with CCl_4 challenging ($P < 0.05$; Table 7). Mean BrG was affected by feed presentation form × CCl_4 interaction so that a greater in the birds fed with the pelleted diet and with no CCl_4 challenge *($P < 0.05$)*. The birds fed with the pelleted and crumble diets with no CCl_4 intoxication showed a plump breast and wider BrA at day 42 of age. Fearfulness assayed by the tonic immobility test was not affected by either feed physical form, CCl_4 injection, or their interction ($P < 0.05$; Table 7).

Table 5. Mean blood serum components of broiler chickens challenged- CCl_4 fed with pellet, crumble, and mash form diet at day 42 of age.

Diet form	CCl_4[1] (mL/kg)	Glucose (mg/dL)	Total protein (g/dL)	Total bilirubin (mg/dL)	Direct bilirubin (mg/dL)	Albumin (g/dL)
Pelleted		254.27	5.56	0.14	0.07	2.23
Crumble		252.70	5.26	0.15	0.06	2.22
Mash		269.27	5.22	0.15	0.06	2.25
SEM		14.38	0.182	0.005	0.003	2.22
	0	253.92	5.25	0.14	0.06	2.23
	1.75	263.49	5.44	0.15	0.07	2.24
	SEM	11.36	0.136	0.004	0.002	0.026
Pelleted	0	229.25^{b}	5.25^{b}	0.13^{c}	0.06^{b}	2.23
Pelleted	1.75	279.29^{a}	5.86^{a}	0.17^{a}	0.08^{a}	2.22
Crumble	0	256.19^{ab}	5.14^{b}	0.14^{ab}	0.06^{b}	2.25
Crumble	1.75	248.21^{ab}	5.37^{ab}	0.15^{ab}	0.06^{b}	2.23
Mash	0	274.32^{a}	5.36^{ab}	0.15^{ab}	0.06^{b}	2.23
Mash	1.75	263.99^{ab}	5.09^{b}	0.16^{a}	0.06^{b}	2.27
SEM		20.34	0.239	0.007	0.004	0.029
P-value						
Diet		0.6728	0.2903	0.4223	0.7534	0.7244
CCl_4		0.6028	0.3463	0.1580	0.4966	0.9315
Diet × CCl_4		0.0498	0.0186	0.0478	0.1579	0.4974

[a,b] Means with different superscript within a column differ significantly ($P < 0.05$). [1]CCl_4 has intraperitoneally injected a dose of 0.5, 0.5, and 0.75 mL/kg body weight diluted in olive oil at a ratio of 1: 1 (v/v) at days 14, 21, and 28 of age.

Table 6. Liver score in broiler chickens challenged with CCl_4 and fed with a diet provided in three pelleted, crumble and mash form at day 42 of age.

Diet form	CCl_4[1] (mL/kg)	Liver score 42 d			
		Score 0	Score 1	Score 2	Score 3
Pelleted		24.56	32.94	36.44	42.31
Crumble		34.60	32.12	30.51	34.39
Mash		38.84	34.94	33.05	23.29
	0	47.37	55.42	44.07	41.41
	1.75	52.63	44.58	55.93	58.59
Pelleted	0	21.43	41.38	37.21	77.42
Pelleted	1.75	78.57	58.62	62.79	22.58
Crumble	0	36.36	52.00	61.11	58.97
Crumble	1.75	63.64	48.00	38.89	41.03
Mash	0	76.19	72.41	35.90	77.42
Mash	1.75	223.81	27.59	64.10	22.58
P-value					
Feed		<.0001	<.0001	<.0001	<.0001
χ^2		114.00	166.00	236.00	198.00
CCl_4		<.0001	<.0001	<.0001	<.0001
χ^2		57.00	83.00	118.00	99.00
Feed × CCl_4		0.0027	0.0544	0.0469	0.0081
χ^2		11.84	5.82	6.11	9.63

[1]CCl_4 were intraperitoneally injected at 0.5, 0.5, and 0.75 mL/kg body weight dosages in olive oil at a ratio of 1: 1, v/v at days 14, 21, and 28 of age.

The tolerance statistic as the main multicollinearity diagnostic was greater than 0.1 for all parameters considered (Table 8). Liver fat percentage showed low phenotypic correlation coefficients (0.2<) with all the recorded parameters except for serum cholesterol and total protein concentration which demonstrated medium positive coefficient correlation of 0.27 and 0.21, respectively (Figure 1).

The best linear model for prediction of liver fat percentage achieved by the Forward modeling approach in SAS involved serum TC, LDL, TP, TBIL, LDH, BrA, and ShL with an R^2=0.3011. The identical linear models generated by both Backward and Stepwise approaches included fear test but external body measurement was removed and their R^2 was lower than the previous model (0.25 in both) (Table 9).

Table 7. Mean external body measurements in broiler chickens challenged with CCl_4 and fed with a diet provided in pelleted, crumble, or mash form at day 42 of age.

Diet form	CCl_4[1] (mL/kg)	Shank length (cm)	Shank width (cm)	Breast length (cm)	Breast width (cm)	Breast girth (cm)	Breast angle (degree)	Fear test (s)
Pelleted		8.50	1.03	10.44	7.13	27.00^{a}	50.55	50.00
Crumble		7.76	1.08	10.09	6.72	25.78^{ab}	48.62	81.00
Mash		7.99	1.02	10.22	6.79	24.71^{b}	49.04	48.00
SEM		0.421	0.044	0.392	0.255	0.446	0.942	18.751
	0	8.36	1.07	10.41	7.18^{a}	26.71^{a}	51.69^{a}	50.05
	1.75	7.81	1.02	10.09	6.58^{b}	24.95^{b}	47.12^{b}	69.01
	SEM	0.344	0.036	0.318	0.208	0.364	0.771	15.345
Pelleted	0	9.21	1.07^{abc}	10.80	7.27^{a}	27.69^{a}	53.09^{a}	45.00
Pelleted	1.75	7.80	0.99^{bc}	10.08	7.00^{ab}	26.30^{ab}	48.01^{bc}	58.04
Crumble	0	7.97	1.02^{abc}	10.36	6.83^{ab}	26.46^{ab}	51.39^{a}	48.12
Crumble	1.75	7.56	1.14^{a}	9.81	6.61^{ab}	25.11^{b}	45.85^{c}	40.95
Mash	0	7.91	1.11^{ab}	10.07	7.44^{a}	26.00^{ab}	50.58^{ab}	48.00
Mash	1.75	8.07	0.92^{c}	10.37	6.13^{b}	23.43^{c}	47.50^{bc}	48.11
SEM		0.344	0.036	0.318	0.208	0.364	0.771	0.153
P-value								
Diet		0.4464	0.5932	0.8115	0.4714	0.0016	0.3182	0.3988
CCl_4		0.2611	0.3267	0.4714	0.0420	0.0007	<0.0001	0.3246
Diet × CCl_4		0.4113	0.0353	0.6106	0.0422	0.0489	0.0214	0.2985

[a,b] Means with different superscript within a column differ significantly ($P < 0.05$). [1] CCl_4 has intraperitoneally injected a dose of 0.5, 0.5, and 0.75 mL/kg body weight diluted in olive oil at a ratio of 1: 1 (v/v) at days 14, 21, and 28 of age, respectively. *SEM*- Standard error of the mean.

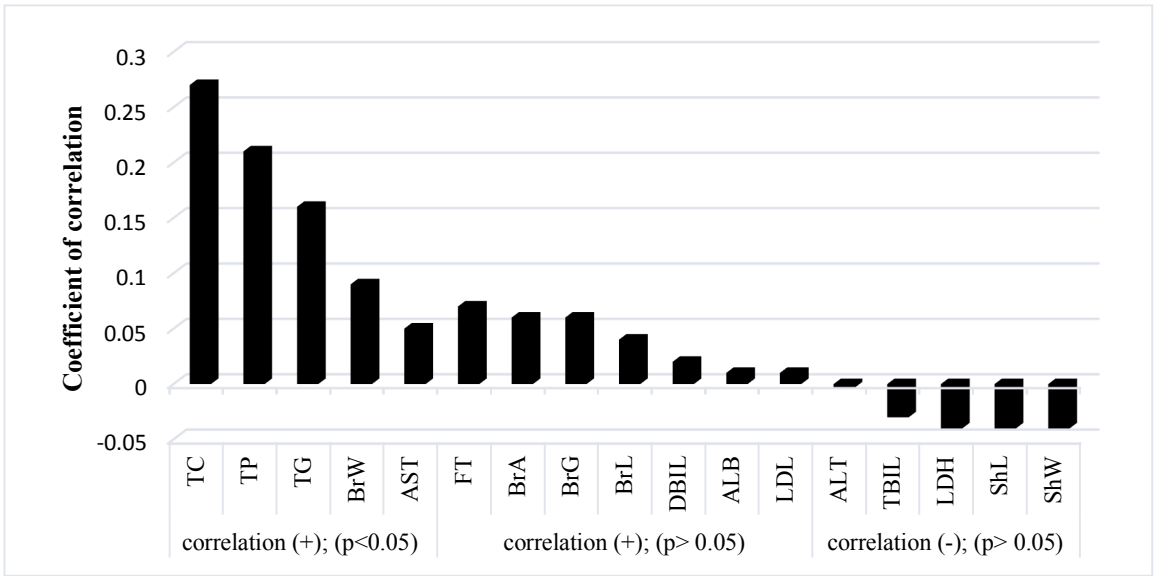

Figure 1. Pearson correlation coefficients between fat liver percentage and blood parameters included; TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; TP, total protein; TBIL, total bilirubin; DBIL, direct bilirubin, ALT, alanine aminotransferase; AST, Aspartate aminotransferase; LDH, lactate dehydrogenase; ALB, albumin and body measurements; ShL, shank length; ShW, shank width; BrL, breast length; BrW, Breast width; BrG, Breast girth; BrA, Breast angle, FT, fear test.

Table 8. The Tolerance (T)[1] estimation to check the collinearity using some of the body measurements and blood parameters in broiler chicken in 42 days of age.

Variable	Symbol	Df	Parameter Estimate	Standard Error	t-value	Prob >t	T
Intercept	-	1	7.82530	7.25042	1.08	0.286	.
Triglyceride	X1	1	0.00311	0.00854	0.36	0.0175	0.30536
Total cholesterol	X2	1	0.04461	0.01463	3.05	0.0039	0.15681
Low-density lipoprotein	X3	1	-0.11172	0.03691	-3.03	0.0041	0.13804
Total protein	X4	1	0.48637	0.44380	1.10	0.2794	0.25423
Total bilirubin	X5	1	0.60422	0.44115	1.37	0.1777	0.27014
Direct bilirubin	X6	1	2.00406	6.03095	0.33	0.7412	0.66139
Alanine aminotransferase	X7	1	0.01876	0.01137	1.65	0.1061	0.21299
Aspartate aminotransferase	X8	1	0.04807	0.09610	-0.50	0.0194	0.62266
Lactate dehydrogenase	X9	1	-0.00060	0.00043	-1.40	0.1688	0.57609
Albumin	X10	1	-3.99078	2.40442	-1.66	0.1041	0.22818
Shank length	X11	1	0.25855	0.79418	0.33	0.7463	0.54683
Shank width	X12	1	-3.38491	3.88329	-0.87	0.3881	0.53304
Breast length	X13	1	-0.44704	0.50583	-0.88	0.3816	0.40261
Breast width	X14	1	0.27215	0.43174	0.63	0.5317	0.46627
Breast angle	X15	1	-0.02206	0.04782	-0.46	0.6469	0.62465
Breast girth	X16	1	0.10405	0.13510	0.77	0.4453	0.46191
fear test	X17	1	2.29260	1.86847	1.23	0.2264	0.76854

[1] The tolerance test was conducted using a without intercept model where liver fat percent considered as an independent variable.

Table 9. Multivariate linear regression equations for prediction of liver fat percentage as a function of blood lipid constituents, certain external body measurements, and fear test in broiler chickens at day 42 of age.

R^2	Variables[1] and coefficients	Approach used
0.3011	Fat=3.62309 + 0.03573 (total cholesterol) - 0.08150 (low density lipoprotein) + 0.46770 (total protein) +4.61280 (total bilirubin) – 0.00091889 (LDH) – 0.03949 (breast angle) +3.18273 (shank length)	Forward
0.2589	Fat= 3.56315 + 0.03619(total cholesterol) - 0.06059 (low density lipoprotein) – 0.00079768 (lactate dehydrogenase) + 2.71582 (Fear test)	Backward
0.2589	Fat= 3.56315 + 0.03619 (total cholesterol) - 0.06059 (low density lipoprotein) - 0.00079768 (lactate dehydrogenase) +2.71582 (Fear test)	Stepwise

Discussion

Almost all poultrymen and researchers believe that a pelleted feed increases weight gain and improves feed efficiency in broiler chickens (Chewning *et al*., 2012; Abdollahi *et al*., 2018). The outcome of the current study, confirm such general idea indicated by increased WG and EPEI and improved FCR in the pelleted feed-fed birds during the whole experimental period. In the line with our results, Mohammadi Ghasem Abadi *et al*. (2019) reported that growth performance is affected interactively by feed form, particle size, and pellet binder. Feeding pellet coarse with 3% pellet binder diets enhanced feed intake and subsequently gained more BW, which confirmed the importance of pellet physical quality. The primacy of a pelleted diet has been attributed to a reduced feed wastage, a decreased time for prehension, an increased realisability of diet ingredients, a declined microbial load among many other advantages (Zang *et al.,* 2009; Abdollahi *et al.,* 2019). However, because of such commercially-privileged merits, the possible disadvantages of a pelleted diet had not been received adequate interest in research. However, in many studies also, feeding pelleted diets did not show any advantage of diets in a crumble or mash form (Shirani *et al*., 2018).

Stressful conditions are an inevitable part of a broiler raising enterprise. As the birds are faced with hectic stimuli such as an extreme environment or contaminated feed with mycotoxins oxidative stress realize as an outcome (Lykkesfeldt and Svendsen, 2007). Intoxication using CCl_4 is a method of choice in animal toxicity models to mimic oxidative stress in experimental organisms (Manibusan *et al.,* 2007; Behboodi *et al*., 2017). Our results showed that DWG reduced and serum concentration of many commonly accepted physiological stress indicators such as lipids and LFP increased in the birds subjected to CCl_4 toxicity compared with the unchallenged birds. Notably, adverse effects intensified in those birds which fed on the pelleted diet than those received the same diet in a crumble of mash form. These findings are in agreement with many previous studies demonstrating the negative effects of CCl_4 on broiler

performance (Sonkusale *et al.,* 2011; Khodadust *et al.,* 2015). Adverse effects of toxins like CCl_4 may initiate in the gut by reducing the small intestine's surface area for absorption (Wang *et al.,* 2018), suppressing protein synthesis through impaired functionality of enzymes (Shuaib *et al.,* 2010), or in a deeper perspective, disrupting function on many internal organs and metabolic pathways. In our study, feeding the pelleted diet worsens the CCl_4 toxicity, indicated by increased LFP and frequency of liver scores 2 and 3 in the birds treated with CCl_4 and fed on the pelleted diet. It was shown that during CCl_4 poisoning fat from adipose tissues is transmitted to the liver (Zhang *et al.,* 2018), leading to fat accumulation, and ultimately resulting in tissue damage (Yalcin *et al.,* 2017), results which has been confirmed by the current study. These findings are also consistent with Moawad (2007) report, who showed that CCl_4 increased serum cholesterol and triglycerides via liver damage. In the CCl_4 treated broilers, the serum content of the total protein and albumin were decreased by 28% and 18%, respectively compared with the control birds. It was also shown that CCl_4 induces lipogenesis through the increased flowing of the acetate toward *de novo* lipogenesis. This process is facilitated via the transfer of acetate into hepatocytes followed by an enhanced blood lipid (Boll *et al.,* 2001).

Several enzymes including ALT, AST, and ALP were shown to demonstrate a greater activity in hepatocytes (Jiang *et al.,* 2015). Therefore, leakage of these enzymes into the bloodstream is anticipated in the pathological destruction of the liver cells (Parmar *et al.,* 2012). In the current study, no significant elevation was observed in the serum activity of ALT, LDH, and ALP. However, Baradaran *et al*., (2019) inconsistent with our results recently reported that CCl_4-treated broilers showed higher serum activity of ALP, AST, ALT, and GGT enzymes compared with birds in the control group, indicating CCl_4-induced hepatotoxicity. Khorramshahi *et al*. (2014) also on the contrary to our results, reported that the Japanese quails treated with CCl_4 intraperitoneally exhibited an elevated serum concentration of ALP, AST, and ALT. Sonkusale *et al.* (2011) and Nateghi *et al.* (2013) also revealed that the administration of CCl_4 to broilers increased the serum activity of the hepatic enzymes. Gad *et al.* (2011) also reported that CCl_4 intoxication decreased serum concentrations of protein and albumin, but increased serum levels for lipid, verifying the adverse effects of CCl_4 on the liver function. The serum concentration of bilirubin, a major product form biotransformation of hemoglobin increases when a liver injury takes place or live damage lead to obstruction of the extrahepatic biliary ducts. Elevation in total serum bilirubin may result from a decreased uptake of and conjugation of bilirubin by the liver as a consequence of hepatic dysfunction (Sanjiv, 2002).

In the current study tonic immobility test as an important welfare criterion was not influenced by experimental treatments. We anticipated increased tonic immobility in the birds exposed to CCl_4 as Ghareeb *et al*. (2014) showed that the intoxication with deoxynivalenol (DON; known as vomitoxin) increased the underlying fearfulness and physiological stress responses in broiler chickens and resulted in a deteriorated welfare status evidenced by elevated plasma concentration of corticosterone, higher H/L ratio and a greater level of fearfulness. Similar reports have been appeared in the literature confirming adverse effects of diary toxins on fearfulness in birds (Roll *et al.,* 2010; Nazar *et al.,* 2012), however, our results may fail to show the same trend due to low CCl_4 doses injected or the administration route.

Whereas outcomes of our study showed a diverged effect of feed physical form on LFP and such diversity was widen by the administration of CCl_4, having a large set of data encouraged us to employ the data for the prediction of LFP in birds. Therefore, in the last part of the experiment, we tried to find a noninvasive way for the prediction of LFP by multivariate linear statistical models to help the clinicians and expert personals in the early finding of the birds suffering fatty liver. We considered 17 predictor parameters for the same purpose. In step 1, a multicollinearity test was conducted and results showed all considered variables were qualified to be included in multivariate linear models as predictor variables because tolerance exceeded 0.1 for all parameters. Tolerance, defined as 1/VIF (variance inflation factor), is applied in several studies to determine the degree of multicollinearity (Yoo *et al.,* 2014). A tolerance value lesser than 0.1 is equivalent to a VIF of 10. It shows that the variable could be considered as a linear combination of other independent variables. As a simple and applicable rule, a variable with a VIF value greater than 10 may merit further investigation or incorporation in statistical models (Hair *et al*., 1995).

In the second step, Pearson correlation coefficients were calculated between all variables and LFP to provide an enhanced understanding of the relationship between the constituents of the models in the next step. No variable was found with a high positive correlation coefficient with LFP, indicating an uncertain prospect to achieve efficient linear models for the planned purpose. Results from this step confirm the previous relevant reports. In a clinical study, Portillo-Sanchez *et al.* (2015) reported that elevated liver enzymes do not show a significant correlation with liver histological grades of fatty liver. Moreover, declined and/or normalized serum concentration of liver enzymes following by an

intervention cannot be a firm indicative criterion for improvement in liver histology. Gerzilov and Petrov, (2015) reported significant positive correlation coefficients between fatty liver weight and blood ALT, AST, and total cholesterol in both genders of chicken. They also demonstrated that blood serum triglycerides, which increased almost twice in liver steatosis, did not correlate with liver weigh.

Despite the mainly weak association between considered variables and LFP, we tried to construct multilinear models. Therefore, considering all predictor variables and using three forward, backward and stepwise modeling approaches in SAS software we reached three multivariate linear models that did not satisfy us because their R^2 could not exceed 0.31. Statistical models could be simple or greatly complicated, but they are always intended to improve our understanding of a system, using the existing data. The most important subsequent influence of response modeling was the theory suggested by Emmans (1981) to predict deliberate food intake in poultry and pigs, which offered a great increase the merit of estimating models by making food intake an output from, as opposed to input to, the growth model (Gous, 2007). In the same way, various biochemical parameters can be used to assess nutritional status including lipid profile and liver enzymes. In the current study, we also subjected our data to multivariable regression analysis to obtain decisive values for the association between the concerned noninvasive blood markers, body measurements, and liver fat. The same method was previously adopted by Takase *et al*. (2017) who predicted liver fat content in human cases by calculating the liver fat index as an alternate marker for fatty liver. Interestingly, tonic immobility time was chosen by two approaches as a significant predictor variable while it had a low correlation with LFP but a high tolerance (0.76). Probably, lack of collinearity with all other predictors in the models was the reason for the incorporation of the same variable. As far as fear is an important surrogate in welfare evaluation in poultry (Abe *et al*., 2013), fear testing may be a component of certification or labeling schemes that incorporate the evaluation of outcome-based welfare criteria on commercial farms. It was shown in several previous works that exogenous toxicants such as aflatoxins affect the tonic immobility duration as a behavioral indicator to stress in broiler chicken (Roll *et al.,* 2010; Nazar *et al.,* 2012).

Conclusion

It was concluded that a broiler diet in a pelleted form improves productive performance in broiler chickens but showed a greater potential to induce fatty liver in the same birds by increasing fat amassing in the liver. More the potential risk of fatty life will increase when feed ingredients are contaminated by environmental or microbial toxicants. In other words, exposure to CCl_4 toxicity imposes adverse effects on broiler performance, blood biochemistry, and certain external body measurements, in particular, when birds are maintained on a pelleted diet. Using all 17 recorded predictor variables, despite no significant multicollinearity, we failed to introduce an efficient linear model for the prediction of LFP because R^2 was not exceeded 0.31 in a model. Attempts have to be made to find more relevant predictors such as liver molecular mediators, cytokines, and transcription factors in serum or implementation of sophisticated nonlinear statistical models for the same purpose.

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