

Article

Effect of Different Levels of L-carnitine and Excess Lysine-Methionine on Broiler Performance, Carcass Characteristics, Blood Constituents, Immunity and Triiodothyronine Hormone

Vincenzo Tufarelli ^{1,*}, Hooman Mehrzad-Gilmalek ², Mehrdad Bouyeh ², Ali Qotbi ², Hossein Amouei ², Alireza Seidavi ^{2,*}, Erwin Paz ³ and Vito Laudadio ³

¹ Department of DETO, Section of Veterinary Science and Animal Production, University of Bari 'Aldo Moro', Valenzano, 70010 Bari, Italy

² Department of Animal Science, Rasht Branch, Islamic Azad University, Rasht 41335-3516, Iran; f.mehrzad88@gmail.com (H.M.-G.); mbouyeh@gmail.com (M.B.); adian2000@yahoo.com (A.Q.); hossein_amouei@yahoo.com (H.A.)

³ Laboratory of Animal Production, Faculty of Agriculture and Forestry, Universidad de La Frontera, Temuco 01145, Chile; e.paz01@ufromail.cl (E.P.); vito.laudadio@uniba.it (V.L.)

* Correspondence: vincenzo.tufarelli@uniba.it (V.T.); alirezaseidavi@iaurasht.ac.ir (A.S.)

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Abstract: The influence of dietary L-carnitine and lysine (Lys)-methionine (Met) levels on productive performance, haematology and triiodothyronine hormone levels of broilers was investigated. Dietary treatments included different L-carnitine (0, 50 and 100 mg/kg) and Lys-Met (0%, 10% and 20% over National Research Council (NRC) (1994) recommendation levels), under a 3 × 3 factorial design arrangement. From the findings, the body weight (BW) gain, feed intake and feed conversion ratio (FCR) of broilers were significantly ($p < 0.05$) influenced by dietary treatments in the different growing stages. Conversely, carcass yield and organs did not differ ($p > 0.05$) among treatments, whereas meat-cuts varied significantly when broilers were fed experimental diets. Moreover, the dietary inclusion of the highest levels of amino-acids (L-carnitine (100 mg/kg) and Lys-Met (+20%)) produced a reduction ($p < 0.05$) of blood uric acid, also leading to the highest triiodothyronine (T3) hormone levels. Based on the obtained results, it can be concluded that the combination of extra levels of L-carnitine associated with Lys-Met positively influenced the performance traits in broiler chickens.

Keywords: diet; broiler; amino acids; diet; thyroxin; meat

1. Introduction

Essential amino acids (AA) are critical for the proper development of poultry. As feed efficiency is crucial factor for intensive rearing systems of poultry, it is imperative to formulate diets that improve and maximize animal production [1,2].

The importance of utilizing the correct amount of balanced dietary AA for poultry is a high priority issue due to its significant impact on broiler growth and meat yield. Therefore, defining dietary AA needs for optimum production is of utmost importance. Essential AA recommendations currently used for broilers by the National Research Council (NRC) Nutrient Requirements of Poultry of 1994 are largely based on results of trials conducted several decades ago [3], and time has passed and scientific advancement has been made since 1994; thus, there is the need for an update poultry NRC guidelines for modern poultry production [3].

L-carnitine (3-hydroxy-4-N-trimethylaminobutyrate) plays a key-role in long-chain fatty acid oxidation by enabling its transport from the cytoplasm to the mitochondrial matrix [4,5]. In broilers, fed diets supplemented with L-carnitine facilitate the beta-oxidation of fatty acids from fat sources, and thus improving productive performance [6,7]. With regard to Lys, it was even reported that Lys in excess of an animal's requirement reduces the concentrations of free and total L-carnitine in tissues. This is most likely explained by the fact that only protein-bound—not free—Lys can be methylated in mammals and avian species. In fact, a large body of evidence has been provided that proves L-carnitine biosynthesis is limited by the availability of free N^ε-trimethyllysine [8,9], which serves as the initial L-carnitine precursor being enzymatically converted into γ -butyrobetaine. Both Lys and Met appear to be the significant limiting amino acids in poultry nutrition, and therefore they must be included in diets [10,11]. The immune role of both amino acids has been studied in many livestock species, involved directly or indirectly in regulatory actions. According to Derballa et al. [12], the deficiency of Lys and Met in fish diets significantly decreased blood lymphocytes, monocytes, basophils and eosinophils levels. Nevertheless, the effect of simultaneous feeding of L-carnitine levels and excess Lys-Met has not been deeply studied.

Therefore, the objective of the present study was to evaluate the effect of different dietary levels of L-carnitine and Lys-Met on productive performance, haematology and the triiodothyronine hormone levels of broilers.

2. Materials and Methods

This experiment was conducted at the poultry farm facilities of the Chukam village, Khomam, Iran and all the procedures were assessed and approved by the Institutional Animal Care and Ethics Committee in Rasht Branch, Islamic Azad University (Rasht, Iran).

Prior to bird allocation, facilities and equipments were disinfected. Five hundred and forty male Ross-308 chicks (Aviagen, Newbridge, Scotland, UK) were assigned randomly to nine different dietary treatments diets with four replications per treatment. Each replication group of 15 mixed birds was reared in 1 × 2 m wire mesh pen. The initial body weight (BW) was similar (44.2 ± 0.8 g) for all broilers. Each cage was equipped with automatic drinkers and a pan feeder. Feed and water were offered ad libitum. A light schedule, temperature, humidity, vaccination, and general management were performed according to the Ross broiler manual [13].

Dietary treatments included: a control treatment (T1) having the levels of Lys-Met according to NRC [14] recommendations (Table 1); whereas the other treatments included extra levels of Lys-Met as: L-carnitine 0, 50 and 100 mg/kg, and Lys-Met 0%, 10% and 20% over the recommendations [14] (T2–T9), under a 3 × 3 factorial design arrangement (Table 2).

Broilers BW and feed intake were evaluated weekly and feed conversion ratio (FCR) was calculated by dividing total feed consumption by BW gain. Carcass measurements were carried out as reported by Jahanpour et al. [15]. Briefly, at 42 days of age, three representative chicks per replicate were selected and sacrificed; then, after slaughter, broilers were eviscerated, and the carcass and different organs and meat-cuts were weighted.

Blood constituent evaluations were carried out following Shabani et al. [16]. Briefly, at day 42, three broilers per group, for a total of 12 subjects per treatment, were selected. Blood was collected from the wing veins into EDTA tubes and immediately transferred to the biochemical and hematological laboratory for analysis. Biochemical analysis, including the levels of plasma glucose, cholesterol, triglyceride, and uric acid were determined using diagnostic kits (Teif-Azmoon Pars Co, Tehran, Iran). The triiodothyronine (T3) hormone determination was carried out according to Seidavi et al. [17].

Analysis of variance under a 3 × 3 factorial design with three L-carnitine treatments (0, 50, and 100 mg/kg in diet) and three Lys-Met treatments (0%, 10% and 20% over NRC recommendations) [14], using a one-way procedure of analysis of variance (ANOVA) was performed. The general linear model (GLM) procedure was used to analyse data (SPSS software version 17.0). Means among groups

were compared by using least significant difference (LSD). The significance level was set at $p < 0.05$. Results are reported as mean \pm standard error of the means (SEM).

Table 1. Ingredients and nutrient composition of basal diets fed to broiler chickens.

Ingredient (g/kg)	Starter	Grower	Finisher
Corn	550.0	571.0	651.0
Soybean meal (44% CP)	386.5	359.0	279.5
Soybean oil	26.0	33.0	33.0
Dicalcium phosphate	15.0	14.0	14.0
Calcium carbonate	11.0	12.0	12.5
Mineral-vitamin premix *	5.0	5.0	5.0
NaCl	2.5	2.5	2.5
Methionine	2.3	2.0	1.5
Lysine	1.2	1.0	0.5
Salinomycin Sodium 12%	0.5	0.5	0.5
<i>Calculated nutrient analysis</i>			
ME (kcal/kg)	2898	2955	3058
Crude protein (%)	21.7	20.8	18.25
Crude fiber (%)	2.26	2.22	2.14
Calcium (%)	0.80	0.80	0.79
Available P (%)	0.48	0.46	0.42
Sodium (%)	0.15	0.15	0.15
Lysine (%)	1.40	1.32	1.22
Methionine (%)	0.61	0.57	0.48
Linoleic acid (%)	2.46	2.73	2.95
Methionine + Cysteine (%)	0.97	0.91	0.77

* Calcium pantothenate 4 mg/g; niacin 15 mg/g; vitamin B₆ 13 mg/g; Cu 3 mg/g; Zn 15 mg/g; Mn 20 mg/g; Fe 10 mg/g; K 0.3 mg/g; vitamin A 5000 IU/g; vitamin D₃ 500 IU/g; vitamin E 3 mg/g; vitamin K₃ 1.5 mg/g; vitamin B₂ 1 mg/g.

Table 2. Levels of L-carnitine, lysine and methionine of the experimental dietary treatments.

	Treatments								
	T1	T2	T3	T4	T5	T6	T7	T8	T9
L-carnitine *	–	–	–	+50 mg/kg	+50 mg/kg	+50 mg/kg	+100 mg/kg	+100 mg/kg	+100 mg/kg
Lysine **	NRC	NRC + 10%	NRC + 20%	NRC	NRC + 10%	NRC+20%	NRC	NRC + 10%	NRC + 20%
Methionine ***	NRC	NRC + 10%	NRC + 20%	NRC	NRC + 10%	NRC+20%	NRC	NRC + 10%	NRC + 20%

* L-carnitine (NRC, 1994): Starter period: 17.8 mg/kg; Grower period: 18.1 mg/kg; Finisher period: 22.9 mg/kg;

** Lysine (NRC, 1994): Starter period 1.41%; Grower period 1.26%; Finisher period 1.22%; *** Methionine (NRC, 1994): Starter period 0.61%; Grower period 0.57%; Finisher period 0.48%.

3. Results

Based on the different levels of L-carnitine and Lys-Met supplied, significant differences among dietary treatments were found in broiler BW gain (Table 3), where supplementing L-carnitine (50 mg/kg) and Lys-Met (+20%) during the grower period led to the best gains ($p < 0.05$). The average feed intake and FCR was significantly affected by treatments, especially during the starter rearing period (1–14 days of age). The feeding of high levels of amino-acids favourably regulated the efficiency of broilers; however, during the grower and finisher periods, feed efficiency was not significantly modified by diets (Tables 4 and 5, respectively). As reported in Table 6, broiler carcass components showed a substantial reduction liver percentage ($p < 0.05$) when fed L-carnitine (50 mg/kg) and Lys-Met (+20%). Supplementing extra levels of L-carnitine and Lys-Met improved ($p < 0.05$) the yield of meat-cuts (breast, drumstick and wings); conversely, no effect was detected in eviscerated carcass yield, heart, pancreas, gizzard and abdominal fat (Table 6).

Table 3. Average body weight gain (g/day) of broilers fed different levels of L-carnitine and lysine-methionine (Lys-Met).

Treatments	1-14 DOA	15-28 DOA	29-42 DOA	1-42 DOA
L-carnitine (0 mg/kg)	18.12	66.43	71.54	52.03
L-carnitine (50 mg/kg)	18.08	65.64	75.90	53.20
L-carnitine (100 mg/kg)	17.57	64.00	69.85	50.48
<i>p</i> -value	ns	ns	ns	ns
Lys-Met (+0%)	17.75	65.89	72.41	52.02
Lys-Met (+10%)	18.01	63.51	71.40	50.97
Lys-Met (+20%)	18.02	66.68	73.48	52.72
<i>p</i> -value	ns	ns	ns	ns
L-carnitine (0 mg/kg) - Lys-Met (+0%)	18.14	68.91 ^{ab}	71.40	52.83
L-carnitine (0 mg/kg) - Lys-Met (+10%)	18.12	67.96 ^{abc}	70.82	52.30
L-carnitine (0 mg/kg) - Lys-Met (+20%)	18.11	62.43 ^{bc}	72.33	50.96
L-carnitine (50 mg/kg) - Lys-Met (+0%)	17.80	64.01 ^{abc}	77.91	53.24
L-carnitine (50 mg/kg) - Lys-Met (+10%)	17.84	62.35 ^{bc}	75.48	51.89
L-carnitine (50 mg/kg) - Lys-Met (+20%)	18.60	70.56 ^a	74.32	54.49
L-carnitine (100 mg/kg) - Lys-Met (+0%)	17.32	64.75 ^{abc}	67.87	49.98
L-carnitine (100 mg/kg) - Lys-Met (+10%)	18.08	60.21 ^c	67.89	48.73
L-carnitine (100 mg/kg) - Lys-Met (+20%)	17.32	67.06 ^{abc}	73.78	52.72
<i>p</i> -value	ns	<0.01	ns	ns
SEM	0.45	2.41	3.88	1.22

Means within each column of dietary treatments with no common superscript differ significantly; DOA: days of age; ns: not significant; SEM: standard error of the means. a–c: means within column differ significantly ($p < 0.05$).

Table 4. Average feed intake (g/day) of broilers fed different levels of L-carnitine and lysine-methionine (Lys-Met).

Treatments	1-14 DOA	15-28 DOA	29-42 DOA	1-42 DOA
L-carnitine (0 mg/kg)	27.26 ^b	113.7	167.1	102.7
L-carnitine (50 mg/kg)	28.21 ^a	113.5	162.8	101.5
L-carnitine (100 mg/kg)	28.28 ^a	106.7	166.6	100.6
<i>p</i> -value	<0.05	ns	ns	ns
Lys-Met (+0%)	27.47 ^b	110.6	168.3	102.1
Lys-Met (+10%)	27.35 ^b	113.8	163.7	101.6
Lys-Met (+20%)	28.95 ^a	109.5	164.6	101.0
<i>p</i> -value	<0.05	ns	ns	ns
L-carnitine (0 mg/kg) - Lys-Met (+0%)	27.66 ^b	112.2	169.2	103.0
L-carnitine (0 mg/kg) - Lys-Met (+10%)	27.45 ^b	114.4	163.7	101.8
L-carnitine (0 mg/kg) - Lys-Met (+20%)	26.67 ^b	114.5	168.4	103.2
L-carnitine (50 mg/kg) - Lys-Met (+0%)	27.5 ^b	111.9	170.3	103.2
L-carnitine (50 mg/kg) - Lys-Met (+10%)	27.38 ^b	113.1	159.5	100.0
L-carnitine (50 mg/kg) - Lys-Met (+20%)	29.73 ^a	115.4	158.6	101.2
L-carnitine (100 mg/kg) - Lys-Met (+0%)	27.23 ^b	107.6	165.4	100.1
L-carnitine (100 mg/kg) - Lys-Met (+10%)	27.22 ^b	113.8	167.8	102.9
L-carnitine (100 mg/kg) - Lys-Met (+20%)	30.39 ^a	98.64	166.7	98.5
<i>p</i> -value	<0.05	ns	ns	ns
SEM	0.741	5.572	10.585	4.969

Means within each column of dietary treatments with no common superscript differ significantly; DOA: days of age; ns: not significant; SEM: standard error of the means. a,b: means within column differ significantly ($p < 0.05$).

Table 5. Feed conversion ratio (g/g) of broilers fed different levels of L-carnitine and lysine-methionine (Lys-Met).

Treatments	1-14 DOA	15-28 DOA	29-42 DOA	1-42 DOA
L-carnitine (0 mg/kg)	1.504 ^b	1.715	2.336 ^{ab}	1.974
L-carnitine (50 mg/kg)	1.559 ^{ab}	1.733	2.145 ^b	1.908
L-carnitine (100 mg/kg)	1.611 ^a	1.647	2.390 ^a	1.995
<i>p</i> -value	<0.05	ns	< 0.05	ns
Lys-Met (+0%)	1.547 ^{ab}	1.680	2.330	1.964
Lys-Met (+10%)	1.518 ^b	1.796	2.299	1.996
Lys-Met (+20%)	1.608 ^a	1.647	2.241	1.918
<i>p</i> -value	<0.05	ns	ns	ns
L-carnitine (0 mg/kg) - Lys-Met (+0%)	1.525 ^b	1.629	2.368	1.950
L-carnitine (0 mg/kg) - Lys-Met (+10%)	1.515 ^b	1.683	2.311	1.947
L-carnitine (0 mg/kg) - Lys-Met (+20%)	1.472 ^b	1.834	2.328	2.025
L-carnitine (50 mg/kg) - Lys-Met (+0%)	1.546 ^b	1.749	2.185	1.939
L-carnitine (50 mg/kg) - Lys-Met (+10%)	1.534 ^b	1.814	2.114	1.927
L-carnitine (50 mg/kg) - Lys-Met (+20%)	1.598 ^b	1.635	2.135	1.858
L-carnitine (100 mg/kg) - Lys-Met (+0%)	1.572 ^b	1.662	2.438	2.003
L-carnitine (100 mg/kg) - Lys-Met (+10%)	1.505 ^b	1.890	2.472	2.113
L-carnitine (100 mg/kg) - Lys-Met (+20%)	1.754 ^a	1.471	2.260	1.870
<i>p</i> -value	<0.05	ns	ns	ns
SEM	0.038	0.149	0.123	0.082

Means within each column of dietary treatments with no common superscript differ significantly; DOA: days of age; ns: not significant; SEM: standard error of the means. a,b: means within column differ significantly ($p < 0.05$).

Table 6. Carcass traits (%) of broilers fed different levels of L-carnitine and lysine-methionine (Lys-Met).

Treatments	Eviscerated Carcass	Breast	Drumsticks	Wings	Liver	Heart	Pancreas	Gizzard	Abdominal Fat
L-carnitine (0 mg/kg) - Lys-Met (+0%)	77.60	22.76 ^{ab}	18.24 ^{ab}	4.86 ^{bc}	2.15 ^a	0.56	0.18	2.20	2.36
L-carnitine (0 mg/kg) - Lys-Met (+10%)	77.43	22.50 ^{ab}	18.37 ^{ab}	5.507 ^{ab}	2.20 ^a	0.57	0.19	2.30	2.10
L-carnitine (0 mg/kg) - Lys-Met (+20%)	77.81	21.35 ^{ab}	18.61 ^{ab}	5.53 ^a	2.18 ^a	0.60	0.21	2.65	2.01
L-carnitine (50 mg/kg) - Lys-Met (+0%)	77.33	21.62 ^{ab}	18.07 ^b	4.96 ^{abc}	2.11 ^a	0.61	0.22	1.91	2.40
L-carnitine (50 mg/kg) - Lys-Met (+10%)	74.57	23.45 ^a	18.92 ^{ab}	5.17 ^{ab}	2.27 ^a	0.52	0.22	2.45	1.78
L-carnitine (50 mg/kg) - Lys-Met (+20%)	76.89	22.52 ^{ab}	18.01 ^b	4.42 ^c	1.49 ^b	0.52	0.19	2.06	2.07
L-carnitine (100 mg/kg) - Lys-Met (+0%)	77.63	22.35 ^{ab}	19.30 ^a	5.28 ^{ab}	2.27 ^a	0.60	0.20	2.53	1.64
L-carnitine (100 mg/kg) - Lys-Met (+10%)	77.32	20.87 ^b	18.67 ^{ab}	5.63 ^a	2.07 ^a	0.57	0.18	2.71	1.81
L-carnitine (100 mg/kg) - Lys-Met (+20%)	77.41	22.19 ^{ab}	18.70 ^{ab}	5.57 ^a	2.39 ^a	0.59	0.15	2.55	2.37
<i>p</i> -value	ns	< 0.05	< 0.05	< 0.01	< 0.05	ns	ns	ns	ns
SEM	0.980	0.661	0.344	0.204	0.148	0.049	0.016	0.257	0.329

Means within each column of dietary treatments with no common superscript differ significantly; DOA: days of age; ns: not significant; SEM: standard error of the means. a–c: means within column differ significantly ($p < 0.05$).

On day 42, some blood parameters were affected by dietary treatments (Table 7). The lowest blood total cholesterol level was found in the control group, whereas the highest was found in broiler fed L-carnitine (100 mg/kg) and Lys-Met (+20%). Furthermore, the feeding of the same diet showed the highest blood uric acid concentration ($p < 0.05$). Blood glucose and triglycerides were not affected by dietary treatments. As reported in Table 8, the blood triiodothyronine (T3) hormone concentration varied significantly among groups, where the highest levels were detected in broilers fed L-carnitine (100 mg/kg) and Lys-Met (+20%) compared to the other treatments; conversely, no difference in spleen percentage was found among dietary groups.

Table 7. Blood constituents (mg/dl) of broilers fed different levels of L-carnitine and lysine-methionine (Lys-Met).

Treatments	Glucose	Total Cholesterol	Triglycerides	Uric Acid
L-carnitine (0 mg/kg) - Lys-Met (+0%)	178.06	113.44 ^d	91.29	6.89 ^a
L-carnitine (0 mg/kg) - Lys-Met (+10%)	207.09	130.20 ^c	94.15	5.31 ^{ab}
L-carnitine (0 mg/kg) - Lys-Met (+20%)	224.25	120.80 ^c	91.30	6.44 ^a
L-carnitine (50 mg/kg) - Lys-Met (+0%)	182.51	116.74 ^c	90.33	6.92 ^a
L-carnitine (50 mg/kg) - Lys-Met (+10%)	187.91	135.07 ^b	63.62	5.57 ^{ab}
L-carnitine (50 mg/kg) - Lys-Met (+20%)	211.01	136.79 ^b	99.51	5.24 ^{ab}
L-carnitine (100 mg/kg) - Lys-Met (+0%)	221.50	129.61 ^c	82.60	6.46 ^a
L-carnitine (100 mg/kg) - Lys-Met (+10%)	226.37	127.40 ^c	91.76	6.09 ^a
L-carnitine (100 mg/kg) - Lys-Met (+20%)	212.38	146.19 ^a	85.98	4.22 ^b
<i>p</i> -value	ns	< 0.01	ns	< 0.05
SEM	19.506	6.036	6.970	0.571

Means within each column of dietary treatments with no common superscript differ significantly; ns: not significant; SEM: standard error of the means. a–c: means within column differ significantly ($p < 0.05$).

Table 8. Immunity and hormonal parameters of broilers fed different levels of L-carnitine and lysine-methionine (Lys-Met).

Treatments	Spleen (%)	T3 (Triiodothyronine, ng/dl)
L-carnitine (0 mg/kg) - Lys-Met (+0%)	0.110	2.125 ^{ab}
L-carnitine (0 mg/kg) - Lys-Met (+10%)	0.102	2.625 ^{ab}
L-carnitine (0 mg/kg) - Lys-Met (+20%)	0.127	1.925 ^{ab}
L-carnitine (50 mg/kg) - Lys-Met (+0%)	0.142	1.725 ^{ab}
L-carnitine (50 mg/kg) - Lys-Met (+10%)	0.107	1.275 ^b
L-carnitine (50 mg/kg) - Lys-Met (+20%)	0.130	2.400 ^{ab}
L-carnitine (100 mg/kg) - Lys-Met (+0%)	0.152	2.100 ^{ab}
L-carnitine (100 mg/kg) - Lys-Met (+10%)	0.087	1.825 ^{ab}
L-carnitine (100 mg/kg) - Lys-Met (+20%)	0.090	3.725 ^a
<i>p</i> -value	ns	< 0.01
SEM	0.023	0.713

Means within each column of dietary treatments with no common superscript differ significantly; ns: not significant; SEM: standard error of the means. a,b: means within column differ significantly ($p < 0.05$).

4. Discussion

Evaluating the different combinations of dietary L-carnitine and Lys-Met, we found considerable differences of broiler performances. In particular, the grower period demands a high synthesis of protein which could explain the significant broiler body weight gain increase by the experimental groups. The greatest improvement in feed intake and feed conversion ratio, was obtained broilers fed the highest levels of L-carnitine and Met-Lys treatments. This is in agreement to a recent study conducted by Ghoreyshi et al. [11]. Conversely, it was suggested that concentrations of Lys and Met above NRC [14] recommendations improve broiler productive traits, such as breast meat yield, body weight gain and feed conversion ratio [18].

Among carcass components evaluated, there were no significant differences, although liver mass displayed a significant increase. The high bioavailability of Lys-Met by diet could intensify the liver functionality to produce L-carnitine. The biosynthesis of L-carnitine takes place in the kidneys and liver from Lys and Met [19]. Both Lys and Met tend to stimulate pancreas function for the further secretion of insulin into the blood. Especially in broilers, insulin promotes the lipoprotein metabolisms and amino-acid release from different bodily sources [20]. A high concentration of insulin and glucagon on plasma had been found in broilers with considerable body weight and fat content [21,22]. The current study showed no significant dietary effect on glucose and triglycerides, whereas the feeding of highest levels of amino-acids (L-carnitine (100 mg/kg) and Lys-Met (+20%)) produced significant less amount of blood uric acid. In this regard, there is a direct relationship of plasma uric acid and excreta uric acid as dietary nitrogen intake increases [23]. As the primary end product of nitrogen metabolism, uric acid, and not urea, is generated in broilers [24]. When daily Lys increased in the broiler diet, plasma uric acid and excreta uric acid decreased [25]. Another investigation has reported no changes in plasma uric acid concentrations after dietary Lys supplementation [24]. Broilers fed the highest levels of L-carnitine and Lys-Met showed a significantly high T3 hormone concentration. Thyroid hormones play a significant role in controlling bird oxidative metabolism; therefore, any pronounced functional changes are expressed in an altered metabolic rate [26].

5. Conclusions

Based on the obtained results, our study demonstrated that the addition of a combination of amino acids (L-carnitine and extra level of Lys-Met) to the diet affected the performance and health status of the broilers chickens positively.

Author Contributions: A.S., H.M.-G. and M.B. conceived and designed the experiments; H.M.-G., M.B., A.Q. and H.A. performed the experiments; A.S., H.M.-G., M.B., E.P. and V.T. analyzed the data; A.S., H.M.-G., V.T., V.L. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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