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Research article

Alleviating the oxidative stress in Japanese quails fed L- carnitine and creatine monohydrate through impacts on productive performance, ileal microflora, digestibility and redox system

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Abstract

This study was aimed to investigate the effects of L-carnitine (Car), creatine monohydrate (CrM) and their combination (CarCrM) as dietary antioxidants materials on productive performance, digestibility, ileal eubiosis, blood chemistry and redox system of stressed quails challenged by lead acetate (LA) in drinking water. In total, 600 quails were assigned into 5 treatments with 4 replications each and 30 chicks per replication from 1 until 42 days old. The treatments involved control (T1), stressed treatment (adding 2.5 ppm of LA in drinking water only; T2) and treatments of adding 500 mg/kg Car+LA (T3), 500 mg/kg CrM+LA (T4) and CarCrM (250 mg/kg Car plus 250 mg/kg CrM)+LA (T5). A completely randomized design was followed to analyze treatment influences on variables. In comparison to T2, the results showed that T5 and T4 had equivalent positive influence followed by T3 to increase ($p \leq 0.05$) body weight, feed intake, survivability, carcass yield and digestibility accompanied with increase lactic acid bacteria and reduced total coliform, *E.coli* in ileum. Also, increased levels ($p \leq 0.05$) of glutathione peroxidase, superoxide dismutase, catalase and glutathione in serum and ferric-reducing ability of plasma were obtained by T5 followed by T4. Moreover, T5 and T4 achieved low values ($p \leq 0.05$) of feed efficiency and serum lipid hydroperoxide, malondialdehyde, cortisol, aspartate transaminase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, creatine kinase and creatinine. The results confirm that dietary CarCrM or CrM mitigated stress and reinforced antioxidant pool which was reflected by supported productive and physiological aspects of birds. Dietary Car seemed less powerful effect than CarCrM and CrM but without negative influence compared with stressed treatment.

Keywords: Antioxidants, Feed additives, Oxidation, Poultry, Production

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INTRODUCTION

It is well known that oxidative stress (OS) at the cellular level is passively connected to poultry welfare and their final productive performance. OS is resulted by high production of free radicals and other reactive species with lowering of antioxidant protection or imbalanced mechanism between the antioxidant and pro-oxidants (redox) in live organisms (Est´evez, 2015; Surai, 2016). The maintenance of multiple physiological reactions and their biochemical pathways which involved immune homeostasis, gene expression and cell signaling is depended upon redox balance in body system of birds (Landy and Kavyani, 2013; Surai et al., 2019; Al-Shammari and Batkowska, 2021). The major injurious consequences of OS are represented by functional damaging of essential elements such as DNA, proteins, carbohydrates and lipid membranes in cells. Some results indicated the role of OS in stimulation of oxidative damage in living tissues through increasing lipid peroxidation with reduction in antioxidant activity network of avian body (Al-Shammari et al., 2019a; Al-Shammari et al., 2019b). Thus, OS could be clearly mitigated by antioxidants substances either by endogenous (*in vivo*) or exogenous (*in vitro*) route which their demand is high during stress status to counteract OS (Kohen and Nyska, 2002; Surai, 2016). At present, there are numerous attempts among researches to find out influential methods to minimize the response to OS status and improve the overall performance of poultry species.

L-carnitine (Car) is water soluble compound which is primarily biosynthesized from methionine and lysine in certain tissues and basically affects mitochondria integrity. Also, some elements are required as cofactors for L-carnitine synthesis such as vitamin C, nicotinic acid, vitamin B_6 and folates (Arslan, 2006). Its biological role is well documented in poultry through increasing growth performance, improving semen quality, strengthening immunity, prevention of diseases and increasing the energy metabolism from fat and glucose origins especially the energy associated with fatty acid β-oxidation via transferring acyl groups to mitochondrial matrix from cytoplasm (Surai, 2015; Rehman et al., 2017). Also, Car has lowered the mortality and susceptibility to ascites due to reduction of right ventricular hypertrophy in heart of broiler reared in high altitude (Yousefi et al., 2013) and low-temperature (Wang et al., 2013) stress. Interestingly, Car acts as powerful antioxidant by immediate scavenging or reduce formation the harmful reactive oxygen species (ROS) through various mode of actions such as synthesis of antioxidative enzymes, deactivating the ROS-generating enzymes, chelating of OS-induced metals and modulating of redox-signaling events (Adabi et al., 2011; Surai, 2015). Poultry needs Car consistently and its requirements are highly increased during growth and egg production circumstances under stressful conditions which depend upon diet protein (Adabi et al., 2011).

Creatine monohydrate (CrM) is an amino acid derivative and one of the major supplement forms of creatine. Creatine is a nitrogen compound which is naturally synthesized in vivo from its immediate precursor (guanidinoacetic acid) (Ibrahim et al., 2019) or from glycine, arginine and methionine in liver. Creatine is received from meat-containing diets and present basically in muscle cells as an energy reserve and turns into phosphocreatine when introduced into animal cell. Phosphocreatine plays its role in ergogenic effects (energy

metabolism) through donating of phosphate group from adenosine diphosphate (ADP) to adenosine triphosphate (ATP) (Wyss and Kaddurah-Daouk, 2000; Wang et al., 2015). Some results reported the pivotal function of creatine to improve productive performance (Carvalho et al., 2013), meat quality and reduction of muscle glycolysis during transport stress (Zhang et al., 2014) with decrease heterocytes/lymphocytes ratio as stress indicator of heat stressed chickens (Al-Tamimi et al., 2019). This is because of palliative effect of creatine to stress response and quenching free radicals with anti-inflammatory properties (Wu, 2020).

According to our information, the potential using of Car and CrM singly or in combination as antioxidant materials proposed in quail diets under OS condition has not been reported yet. Therefore, the current study was aimed to use these additives individually or synergistically in diet and tested for their potential antioxidative properties with a specific emphasis to their influence on productivity, biochemical indices, redox status, ileal microbes and digestibility for meat-type Japanese quails during OS stimulated by lead acetate provided in drinking water.

MATERIALS AND METHODS

Ethics Approval

This experiment was implemented at the Poultry Farm and Labs of the Al-Musaib Technical College, Al-Furat Al-Awsat Technical University, Babylon, Iraq. All the procedures were approved and assessed in congruence with the relevant guidelines of Scientific Committee in Department of Animal Production Techniques-Al-Musaib Technical College based on the Iraqi legislation and instructions issued under the amended law No.22 of 1972 about regulating the slaughter of animal and other animal welfare regulations.

Experiment Treatments and Birds Management

In total, 600 mixed sex Japanese quail chicks were distributed randomly to 5 treatments with 4 replications per treatment and each replication contained 30 chicks with similar body weight (6.56±0.3) for all chicks. Birds were reared for 6 weeks in wire cage system (60×90 cm) equipped with feeders and drinkers with provision all environmentally controlled conditions and *ad libitum* feeding on unified balanced diet based on yellow corn and soybean meal (NRC, 1994; Table 1). Temperature was set at 34°C during the first week of rearing and reduced by 2.5°C a week to maintain 24°C until the end of experiment at 6 weeks. The photoperiod was provided at 23 hours per day for whole experiment. The treatments included a control treatment with no stress and feed additives (T1), stressed treatment which stimulated by 2.5 ppm lead acetate (LA) in drinking water (T2); whereas the other stressed treatments were involved adding 2.5 ppm LA in drinking water plus adding 500 mg/kg L-carnitine (Car) (T3), 500 mg/kg creatine monohydrate (CrM) (T4) and mixture of 250 mg/kg Car and 250 mg/kg CrM (T5) in diet. LA was in commercial powder form (99% purity, Lab Tech Chemicals, Germany) used as stressful substance (pro-oxidant factor) in drinking water to provoke the OS in *vivo* experimentally (Mehrotra et al., 2008; Al-Shammari, 2017; Al-Shammari and Batkowska, 2021). The prepared

LA solution was added to drinking water and kept in tightly closed big plastic container to preserve its oxidative purity and offered to birds daily. Both feed additives, Car and CrM were also procured from commercial source (Shandong Longchang Animal Health Product Co., Ltd/China) in powder form and tested as antistress materials in diet individually or in combination (CarCrM). After mixing these additives in fresh feed according to the tested addition, all diets were kept away from the detrimental effects of sunlight through packaging in polypropylene woven sacks and were provided to birds three times daily.

Table 1 Basal diet and chemical content of its constituents

Feedstuff (%)	Chemical analysis**	Feedstuff (%)	Chemical analysis**
soybean meal	38.69		
yellow corn	53.03	crude protein (%)	24.0
sunflower oil	1.670	metabolizable energy (kcal/kg)	2901
corn gluten	3.200	lysine (%)	1.59
L-lysine	1.500	methionine (%)	0.50
DL-methionine	0.390	methionine + cysteine (%)	0.80
limestone	0.300	crude fiber (%)	3.90
dicalcium phosphate	0.810	calcium (%)	0.83
premix*	0.300	available phosphorus (%)	0.42
sodium chloride	0.110	ether extract (%)	4.23
Total	100.00		

* Premix (Provimi 3110, Jordan) supplies per kilogram of diet the following: 7% crude protein, 3800 kcal metabolizable energy, 1.1% fat, 4% lysine, 8.0 % methionine, 8.5% methionine+cysteine, 0.55% threonine, 57500 IU vitamin A, 3000 mg vitamin E, 138 mg vitamin K3, 20125 IU vitamin D3, 138 mg vitamin B1, 345 mg vitamin B2, 1840 mg vitamin B3, 552 mg vitamin B5, 46 mg vitamin B9, 1000 mg vitamin B12, 184 mg vitamin B6, 5.4% calcium, 4.8% sodium, 50 mg iodine, 20000 mg iron, 3680 mg zinc, 3680 mg manganese, 11% phosphorus, 2760 mg choline chloride, 9.2 mg selenium and 6900 g biotin.
** Calculated based on NRC (1994).

Studied Parameters

Growth Performance

Body weight (BW), body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), water intake, survivability were registered daily and presented periodically from 1-3, 4-6 and 1-6 weeks. Likewise, protein efficiency ratio (PER) and energy efficiency ratio (EER) were calculated by dividing periodical weight gain by protein intake and energy intake, respectively. Production efficiency factor (PEF) was calculated in whole period of rearing at 6 weeks based on Lemme et al. (2006). Carcass quality was measured at the end of experiment (6 weeks of age) by taking and selecting 4 birds (1 male, 3 females) per replicate (n=16 /treatment). These birds were weighed accurately before and after being slaughtered by sharp knife, then decapitated, defeathered and eviscerated to calculate proportional weight of carcass, carcass parts (breast, thighs, wings, trunk) and abdominal fat content. From the slaughtered birds, the edible organ (gizzard, liver, heart) were removed and weighed individually using digital scale with an accurate-sensitivity to obtain their weights in relation to live BW.

Gut Length and Weight

Each part of digestive system starting from the esophagus till rectum was weighed accurately to calculate the total gut weight in relation to live BW. Moreover, tape measure and digital vernier caliper (Meter-ISO, China) was used to calculate total gut length.

Apparent Nutrient Digestibility

To determine apparent nutrient digestibility, 80 birds (4 per replicate) were randomly chosen and placed in individual metabolism cages at 6 weeks of age. The digestibility determination was carried out after 5 days period of total excreta collection. After that, the collected excreta were homogenized and dried overnight at 60 ˚C in each replicate as pooled sample. Prior to proximate analyses, excreta of different treatments were ground through a 0.5 mm mesh screen. Kjeldahl method was followed to determine crude protein and all protocols stated by the AOAC (2001) were done to analyze dry matter, ether extract, crude fiber, nitrogen free extract and crude ash. The nutrient digestibility was calculated using the formula coined by Ratriyanto et al. (2020).

Ileal Microbial Population

From the same birds per replicate (n=2/replicate) which bled for carcass quality determination, they were subjected for microbial examination at the end of the experiment, the ileal contents were extracted separately from each replicate and collected gently by squeezing into the sterile tubes. The method of Bryant and Burkey (1953) was followed, briefly, before inoculation of microbial isolations onto Petri dishes with sterile agar, a serial dilution (10^{-4} to 10^{-6}) of ileal collections in anaerobic diluents was performed. The diluents were prepared from 9 ml phosphate buffer solution in sterile test tubes. Before being transferred to the microbial analysis for processing under refrigeration, the quantity of 1 g of ileum contents were taken by sterile swab, homogenized for 3 min with 10 ml of sterile saline peptone water and then mixed under aseptic conditions. 1 ml subtracted from the 10 ml of buffer and ileal content solution was transferred and thoroughly mixed to each tube in replicate to achieve a ratio1:9 dilution. Later, 1 ml of each test tube was added to Petri dishes with the selective culture. Eosin methylene blue agar, *Shigella* agar, McConkey agar and MRS agar (Merck, Darmstadt, Germany) were used to grow and enumerate of *E. coli, Salmonella* spp, coliforms and lactic acid bacteria, respectively after the aerobical incubation of Petri dishes for 24h at 37°C (Manafi et al., 2016). After inoculation between 24 and 48h, the bacterial colonies plates were examined for their specific colony type. The total bacteria count was enumerated and determined after 48 h incubation at 30°C on plate count nutrient agar (Merck, 1.05463) while yeasts and molds (total fungal counts) were counted after being incubated at 25°C for 5 days (Speak, 1984) by using Rose-Bengal chloramphenicol agar. Finally, all colonies plates were manually enumerated, adjusted and converted to log 10 colony forming units per gram of ileum content (log10 CFU/g).

Blood Sampling, Oxidative Stress Markers and Biochemical Values

Blood samples were harvested from 16 birds per treatment (1 male, 3 females /replicate) by jugular vein directly after slaughtering at 6 weeks of age to obtain ample amounts of blood. Blood samples (4 ml/bird) were collected into gel separator tube and centrifuged for 15 min at 3000 RPM to separate serum, and then all samples were kept at –25°c until biochemical analyses. The markers of serum lipid peroxidation which included lipid hydroperoxide (LOOH) and malondialdehyde (MDA) were measured based on procedures of Södergren et al. (1998) and Salih et al. (1987), respectively. Also, the antioxidative enzymes involving catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined according to the protocols described by Misra and Fridovich (1972) and Aebi (1984). All analyses for oxidation indicators in serum stated above were conducted by using commercial kits (Sigma Aldrich, St. Louis, MO, USA) with run a spectrophotometer (Shenzhen, China). Glutathione (GSH) concentration was measured by using the quantitative colorimetric method through following the instructions mentioned in Bioxytech GSH-420, USA) kit. For determination of serum cortisol, a specific enzyme-radioimmunoassay kit (IDS, Boldon, UK) was used for analysis by following the illustrated steps of manufacturer company with using ELISA microplate reader (BK-EL10C, Biobase, USA) to record the absorbance. Part of blood collection (2 ml/bird) was preserved into anticoagulant K_3EDTA tubes to achieve the plasma for calculation ferric-reducing ability of plasma (FRAP) based on the procedure of Benzie and Strain (1996). For determination of biochemical values in serum, diagnostic bioassay kits (Biolabo, French) was used for creatinine, uric acid and alkaline phosphatase (ALP) determination (Burtis and Ashwood, 1999). Glucose value was determined using Cromatest,Spanish kit based on method coined by Young (2000). Alanine aminotransferase (ALT) and aspartate transaminase (AST) activity was measured using Randox enzymatic kit (English) following o Reitman and Frankel (1957). Analysis of gamma-glutamyl transferase (GGT) and creatine kinase (CK) activity was performed spectrophotometrically using standard methods of their accompanying kit (Ortho-Clinical Diagnostics, USA) based on protocol reported by Tietz (1986).

Statistical Analysis

This experiment was performed with a completely randomized design and all collected data were subjected to the ANOVA using the general linear model (GLM) procedure of SAS software for Windows (SAS, 2012). Duncan's multiple range test (Duncan, 1955) was used to compare the statistical significance among the means based on significance level of $P \leq 0.05$ and the mathematical model used was as follows: $Yij = \mu + Ti + eij$. where Yij, observation; μ, overall mean; Ti, treatment influence (1–5) and eij, random error.

RESULTS

Table (2) shows that BW and BWG were improved significantly ($p \leq 0.05$) in T4, T5 and T1 from 1-3 and 6 weeks and high BWG was for T3, T4 and T1 from 4-6 weeks compared to T2. High feed intake ($p \leq 0.05$) was registered in T4 and T1 at 1-3 weeks whereas high values ($p \leq 0.05$) in this trait were in T3 and T1 at 4-6 weeks and in T3, T4 and T1 at 1-6 weeks. Regarding the water intake, there was increase ($p \leq 0.05$) in favor of T5 and T1 at 1-3 weeks, increase for all stressed groups at 4-6 weeks with increase for T3, T5 and T1 at 1-6 weeks compared with T2. The T4, T5 and T1 achieved low average ($p \leq 0.05$) in FCR (1-6 weeks) and high EER (1-3 weeks) compared with T2. Also, in comparison to T2, there was increase ($p \leq 0.05$) in PER at 1-3 weeks for T5 and T1. No significant differences among groups were observed from 4-6 weeks concerning the FCR, PER and EER. All experimental groups and T1 had high ($p \leq 0.05$) periodical survivability and PEF compared with T2.

Table 2 Productive performance of stressed Japanese quail influenced by dietary Car,CrM and CarCrM.

Traits	Age (weeks)	Treatments					SEM	P-value
		T1	T2	T3	T4	T5		
BW (g)	1-3	89.42^{a}	74.53^{c}	82.73^{bc}	85.43^{ab}	88.28^{a}	8.870	0.023
	1-6	157.75^{a}	130.33^{b}	148.38^{ab}	152.28^{a}	150.53^{a}	12.36	0.047
BWG (g)	1-3	82.86^{a}	67.97^{b}	76.17^{ab}	78.87^{a}	81.72^{a}	6.281	0.024
	4-6	68.10^{a}	55.34^{b}	65.52^{a}	66.62^{a}	62.04^{ab}	4.981	0.042
	1-6	151.19^{a}	123.78^{b}	141.81^{ab}	145.72^{a}	143.96^{a}	28.98	0.050
feed intake (g)	1-3	181.18^{a}	168.21^{b}	175.27^{ab}	178.52^{a}	174.22^{ab}	32.81	0.025
	4-6	556.41^{a}	519.51^{b}	545.61^{a}	539.52^{ab}	520.10^{b}	19.87	0.037
	1-6	738.29^{a}	688.72^{b}	720.84^{a}	718.19^{a}	694.29^{ab}	15.23	0.018
water intake (ml)	1-3	391.22^{a}	338.18^{b}	363.94^{ab}	349.04^{b}	367.33^{a}	18.85	0.024
	4-6	1088.11^{a}	938.51^{b}	1078.42^{a}	1049.16^{a}	1044.12^{a}	27.98	0.046
	1-6	1479.5^{a}	1277.4^{b}	1442.6^{a}	1398.3^{ab}	1411.5^{a}	12.66	0.029
FCR (g/g)	1-3	2.18	2.47	2.30	2.26	2.13	0.121	0.077
	4-6	8.06	8.91	8.24	8.10	8.15	1.642	0.092
	1-6	4.88 b	5.56^{a}	5.08^{ab}	4.92^{b}	4.82^{b}	0.280	0.050
PER (g/g)	1-3	1.91^{a}	1.68^{b}	1.82^{ab}	1.84^{ab}	1.95^{a}	0.151	0.017
	4-6	0.53	0.44	0.50	0.51	0.49	0.012	0.062
	1-6	0.85	0.74	0.81	0.84	0.86	0. 112	0.069
EER (g/100 kcal)	1-3	15.76^{a}	13.92^{b}	14.98^{ab}	15.22^{a}	16.16^{a}	1.321	0.043
	4-6	4.22	3.69	4.14	4.26	4.12	2.070	0.073
	1-6	7.05	6.19	6.78	6.99	7.14	0.542	0.092
Surv (%)	1-3	99.16^{a}	89.16^{c}	96.66^{ab}	95.00^{b}	97.50^{a}	1.871	0.048
	4-6	98.32^{a}	91.75^{c}	95.71^{ab}	94.78^{b}	97.44^{a}	7.987	0.020
	1-6	97.50^{a}	81.66^{c}	92.50^{ab}	90.00^{b}	95.00^{a}	2.970	0.038
PEF	1-6	7.49^{a}	4.55^{b}	6.42^{a}	6.62^{a}	7.06^{a}	1.481	0.018

T1: control treatment, T2: stressed treatment by adding 2.5 ppm LA in drinking water; T3, T4 and T5: treatments of adding 500 mg/kg Car, 500 mg/kg CrM and mixture of 250 mg/ kg Car + 250 mg/kg CrM (CarCrM) in diet, respectively under stress condition by 2.5 ppm LA in drinking water. SE: standard error mean. a, b: means within rows with different superscripts differ significantly at $p \leq 0.05$.

BW: body weight, BWG: body weight gain, FCR: feed conversion ratio, PER: protein efficiency ratio, EER: energy efficiency ratio, Surv: survivability, PEF: production efficiency factor.

Based on data represented in Table 3, it was clear that T4, T5 and T1 registered increase ($p \leq 0.05$) in carcass yields and gut length with no significant differences among groups in proportional weights of breast, thighs, wings, trunk, heart and gizzard. However, low ($p \leq 0.05$) relative weight of abdominal fat and high ($p \leq 0.05$) relative weight of liver and gut was in favor of all experimental groups compared with T2.

Table 3 Carcass quality and gut relative weight and length of stressed Japanese quail influenced by dietary Car,CrM and CarCrM.

Traits	Treatments					SEM	P-value
	T1	T2	T3	T4	T5		
carcass yield[1] (%)	70.16[a]	64.21[b]	66.5[ab]	67.43[a]	68.84[a]	9.090	0.047
carcass yield[2] (%)	75.26[a]	69.23[b]	72.65[ab]	73.23[a]	74.19[a]	3.980	0.032
breast (%)	34.34	32.44	34.28	34.62	34.52	3.431	0.064
thighs (%)	23.23	24.32	23.5	24.73	24.31	2.321	0.081
wings (%)	13.83	13.55	13.23	13.15	13.64	1.542	0.065
trunk (%)	28.64	29.27	28.93	27.42	27.5	3.762	0.072
abdominal fat (%)	1.31[b]	2.97[a]	1.27[b]	1.39[b]	1.01[b]	0.123	0.050
heart (%)	1.81[a]	1.39[b]	1.57[ab]	1.46[ab]	1.52[ab]	0.108	0.035
liver (%)	1.95[a]	1.46[b]	1.96[a]	1.99[a]	1.97[a]	0.071	0.026
gizzard (%)	1.84	1.65	1.97	1.75	1.71	0.122	0.093
gut weight (%)	7.88[a]	6.48[b]	7.76[a]	7.32[a]	7.84[a]	1.121	0.032
gut length (cm)	86.78[a]	80.89[b]	83.03[ab]	84.49[a]	85.48[a]	2.370	0.041

T1: control treatment, T2: stressed treatment by adding 2.5 ppm LA in drinking water; T3, T4 and T5: treatments of adding 500 mg/kg Car, 500 mg/kg CrM and mixture of 250 mg/ kg Car + 250 mg/kg CrM (CarCrM) in diet, respectively under stress condition by 2.5 ppm LA in drinking water. SE: standard error mean. a, b: means within rows with different superscripts differ significantly at $p \leq 0.05$.

[1]: without giblets, [2]: with giblets.

Table 4 declares that the best ($p \leq 0.05$) apparent digestibility in dry matter, crude protein, ether extract and nitrogen free extract was obtained by all experimental groups and T1 compared with T2. Apparent digestibility of crude fiber and crude ash was improved ($p \leq 0.05$) in T4, T5 and T1 in comparison to T2.

It was obvious from Table 5 that were no significant differences among groups in counting of total bacteria, *Salmonella* spp and total fungi in ileum. However, high ($p \leq 0.05$) lactic acid bacteria were found in T3, T5 and T1 compared with T2 whereas decrease ($p \leq 0.05$) in total coliform and *Escherichia coli* was for T4, T5 and T1 compared with T2.

Table 4 Apparent nutrient digestibility (%) of stressed Japanese quail influenced by dietary Car,CrM and CarCrM.

Traits	Treatments					SEM	P-value
	T1	T2	T3	T4	T5		
dry matter	80.76^{a}	73.86^{b}	78.87^{a}	79.45^{a}	79.43^{a}	21.453	0.037
crude protein	78.76^{a}	70.76^{c}	74.76^{b}	77.43^{ab}	78.65^{a}	16.484	0.025
ether extract	87.36^{a}	76.85^{c}	83.38^{ab}	82.54^{b}	85.36^{ab}	22.872	0.029
crude fiber	34.42^{a}	31.53^{b}	32.47^{ab}	34.62^{a}	32.86^{a}	12.763	0.041
nitrogen free extract	88.32^{a}	74.25^{c}	84.37^{ab}	83.74^{b}	86.36^{a}	15.475	0.033
crude ash	72.87^{a}	66.76^{b}	67.98^{ab}	72.26^{a}	71.81^{a}	8.812	0.016

T1: control treatment, T2: stressed treatment by adding 2.5 ppm LA in drinking water; T3, T4 and T5: treatments of adding 500 mg/kg Car, 500 mg/kg CrM and mixture of 250 mg/ kg Car + 250 mg/kg CrM (CarCrM) in diet, respectively under stress condition by 2.5 ppm LA in drinking water. SE: standard error mean. a, b: means within rows with different superscripts differ significantly at $p \leq 0.05$.

Table 5 Ileal microflora population (log_{10} cfu/g of wet weight) of stressed Japanese quail influenced by dietary Car,CrM and CarCrM.

Traits	Treatments					SEM	P-value
	T1	T2	T3	T4	T5		
total bacteria	8.26	9.49	8.66	8.18	8.22	3.986	0.082
lactic acid bacteria	5.87^{a}	4.15^{b}	5.34^{a}	4.32^{ab}	5.64^{a}	1.767	0.041
total coliform	3.03^{b}	5.28^{a}	4.48^{ab}	3.16^{b}	3.17^{b}	1.172	0.027
Escherichia coli	2.28^{b}	4.97^{a}	3.13^{ab}	2.46^{b}	2.14^{b}	1.565	0.035
Salmonella spp	4.19	5.17	4.23	4.32	4.26	1.635	0.069
total fungi	3.18	4.43	3.53	3.75	3.48	1.593	0.058

T1: control treatment, T2: stressed treatment by adding 2.5 ppm LA in drinking water; T3, T4 and T5: treatments of adding 500 mg/kg Car, 500 mg/kg CrM and mixture of 250 mg/ kg Car + 250 mg/kg CrM (CarCrM) in diet, respectively under stress condition by 2.5 ppm LA in drinking water. SE: standard error mean. a, b: means within rows with different superscripts differ significantly at $p \leq 0.05$.

Regarding the stress indicators (Table 6), high ($p \leq 0.05$) GPx value was in favor of T3, T5 and T1 compared with T2. High ($p \leq 0.05$) SOD was for T4, T5 and T1. All experimental groups had high ($p \leq 0.05$) levels in CAT and GSH and low ($p \leq 0.05$) LOOH compared with T2. T4, T5 and T1 registered increase ($p \leq 0.05$) in FRAP with decrease ($p \leq 0.05$) in MDA for the same treatments in comparison to T1. Lowering ($p \leq 0.05$) was found only for T5 and T1 in cortisol value compared with T2.

Table 6 Stress indicator in stressed Japanese quail blood influenced by dietary Car,CrM and CarCrM.

Traits	Treatments					SEM	P-value
	T1	T2	T3	T4	T5		
GPx (U/l)	1.98^{a}	1.21^{b}	1.87^{a}	1.24^{b}	1.86^{a}	0.762	0.027
SOD (U/ml)	257.13^{a}	199.34^{b}	238.12^{ab}	240.23^{a}	243.32^{a}	76.98	0.042
CAT (U/ml)	687.3^{a}	487.3^{c}	501.4^{b}	587.2^{ab}	624.4^{a}	47.87	0.036
GSH (µmol/l)	68.54^{a}	43.65^{c}	50.43^{b}	59.87^{ab}	62.98^{a}	21.65	0.049
FRAP (µmol/l)	343.32^{a}	210.12^{c}	298.42^{bc}	319.26 ^{ab}	322.43^{a}	94.87	0.023
LOOH (µmol/l)	32.87^{c}	47.76^{a}	39.87^{b}	38.98^{b}	35.42b^{c}	8.761	0.021
MAD (µmol/l)	2.15^{b}	3.47^{a}	3.26^{a}	2.34^{b}	2.19^{b}	1.872	0.046
cortisol (µg/dl)	0.04^{b}	0.12^{a}	0.07^{ab}	0.06a^{b}	0.05^{b}	0.002	0.030

T1: control treatment, T2: stressed treatment by adding 2.5 ppm LA in drinking water; T3, T4 and T5: treatments of adding 500 mg/kg Car, 500 mg/kg CrM and mixture of 250 mg/ kg Car + 250 mg/kg CrM (CarCrM) in diet, respectively under stress condition by 2.5 ppm LA in drinking water. SE: standard error mean. a, b, c: means within rows with different superscripts differ significantly at p≤0.05.
GPx: glutathione peroxidase, SOD: superoxide dismutase, CAT: catalase, GSH: glutathione, FRAP: ferric-reducing ability of plasma, LOOH: lipid hydroperoxide, MDA: malondialdehyde.

The results found in Table 7 refers that was reduced glucose (p≤0.05) for T1 compared with other groups. Considerable lowering (p≤0.05) in ALP level for T3, T4, T5 and T1 compared with T2. The decrease (p≤0.05) in AST activity was noted for T4, T5 and T1. Levels of creatinine, ALT and GGT were decreased (p≤0.05) for T5 and T1 compared with T2. In comparison to T2, low CK activity was in favor of T3, T5 and T1.

Table 7 Biochemical values in stressed Japanese quail blood serum influenced by dietary Car,CrM and CarCrM.

Traits	Treatments					SEM	P-value
	T1	T2	T3	T4	T5		
glucose (mg/dl)	200.54^{b}	214.63^{a}	213.32^{a}	210.31^{a}	203.62^{ab}	10.54	0.020
creatinine (mg/dl)	1.19^{b}	1.50^{a}	1.32^{ab}	1.43^{a}	1.29^{b}	0.540	0.019
uric acid (mg/dl)	4.32	5.13	4.15	4.55	4.06	1.320	0.084
AST (U/l)	142.51^{c}	157.73^{a}	155.52^{a}	148.71^{b}	145.41^{bc}	35.65	0.038
ALT (U/l)	23.42^{b}	33.38^{a}	32.42^{a}	28.34^{ab}	26.46^{b}	9.870	0.041
ALP (U/l)	154.34^{c}	200.64^{a}	160.45^{b}	162.12^{b}	158.33^{bc}	34.43	0.035
GGT (U/l)	5.11^{b}	8.53^{a}	6.87^{ab}	7.31^{a}	6.65^{b}	1.650	0.027
CK (U/l)	136.25^{c}	146.26^{a}	139.34^{bc}	143.62^{ab}	137.96^{c}	43.65	0.030

T1: control treatment, T2: stressed treatment by adding 2.5 ppm LA in drinking water; T3, T4 and T5: treatments of adding 500 mg/kg Car, 500 mg/kg CrM and mixture of 250 mg/ kg Car + 250 mg/kg CrM (CarCrM) in diet, respectively under stress condition by 2.5 ppm LA in drinking water. SE: standard error mean. a, b: means within rows with different superscripts differ significantly at p≤0.05.
AST: aspartate transaminase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, GGT: gamma-glutamyl transferase, CK: creatine kinase.

DISCUSSION

Enhanced productive performance (Table 2) and carcass yields with reduced abdominal fat (Table 3) of birds fed CrM (T4) and CarCrM (T5) followed by Car (T3) which exposed to OS by LA in drinking water compared to T2 (stressed birds) could justify the significant importance of these additives to alleviate the stress condition. This is probably that creatine (Cr) as effective antioxidant factor characterized by its readily absorption and widely distribution into cellular sarcoplasm to decrease muscle glycolysis (Lawler et al., 2002). Also, Cr and its analogs exert growth-promoting effects by motivation the digestion process and metabolic profiles of energy through modulating energy demands and provide the protection for muscular membranes from vulnerable influence with promoting pancreatic secretions (Wyss and Kaddurah-Daouk, 2000). Regarding improved BW, BWG and carcass yields might be due to influence of CrM on up-regulated gene expression levels of myogenin, insulin like growth factor-1 and growth hormone which are the directly associated with myoblast differentiation, muscle fibers growth and myostatin downregulating in pectoral and leg muscles and hepatic tissue which are in turn reflect on increased overall BW (Chen et al., 2011; Ibrahim et al., 2015; Ibrahim et al., 2019). Also, ameliorative effect in T4 could be attributed to efficiency of CrM to reduce rapid muscular glycolysis and increase ATP levels and this could preserve the muscular synthesis via molecular mechanism of inhibition and downregulating of liver kinase B1 (LKB1) and adenosine 5'-monophosphate-activated protein kinase α2 (AMPKα2) which are involved in regulating metabolic growth, improving buffering capacity of body energy in stressed chickens (Zhang et al., 2017). Also, high FI in CrM is probably correlated with increased levels of leptin and pancreatic hormones in plasma which involved in stimulation and regulation of FI of broiler (Chen et al., 2011a). The positively slight increase in BW of T3 could explain the importance of Car in upregulation of gene expression for myogenic determining factor, myogenic factor 5 and cationic amino acid transporter which are related to muscle differentiation, myogenesis and growth in chickens (Abouzed et al., 2019). High FI in T3 could be associated with positive influence of Car to enhance villus height and ratio of villus height/crypt depth in duodenum which considered as crucial marker to stimulate digestion, absorption of nutrients which are in turn increase FI and improved FCR in quail (Mahmoud et al., 2020). Another possible reason to increase FI in stressed birds (T3, T4 and T5) is probably due to increased proportional weight of gut and absolute gut length (Table 3) which could encourage to occupy more feed in digestive system. Meanwhile, T5 was superior to T3 in improve FCR which explain the synergistic effect of Car instead of its individual using in diet for secretory stimulation of digestive enzymes involving trypsin, amylase, lipase and chymotrypsin in small intestine which markedly elevate the resbsorption of essential minerals for physiological process in quails body (Abdel-Fattah et al., 2014). High amount of water intake in T3, T4 and T5 could be speculated that any reason causes the toxic or stressful stimulation may lead to increase water consumption as attempt to palliate the OS and decrease levels of the prooxidant (LA) retained in tissues by accelerate its excretion through liver and

kidney (Mehrotra et al., 2008) and this result was recently confirmed by Al-Shammari and Batkowska (2021). Thus, there was increased relative weight of liver in those groups (Table 3). Increased values of PEF as final economic profit indicator of experiment in stressed birds groups and T1 might be as a result of overall increase in productive performance which basically depend upon final BW, mortality and FCR (Lemme et al., 2006). There is absence of data related to using CrM in quails feeding, however, our findings are harmonious with Carvalho et al. (2013) who revealed that inclusion 600g/ton Cr+5% blood meal in male chicks diets improved BW and FCR with no effect on feed intake, carcass yield and proportional weight of boneless leg and breast compared to inclusion of 5% blood meal in diet at 42 day. Also, it was concluded that addition of Cr in drinking water at 0.25 and 0.5 g/kg for broiler chickens and rats has increased BWG but without differences in FCR at 15 days old (Moghadam et al., 2008). Different results are obtained by Zhang et al. (2014) who reported that 600 and 1200 mg/kg CrM supplemented in diet of broiler males for 28 days lasted from 14 days till 42 days could alleviate the transport stress for 3 hours but did not show any changes in daily FI, BW, BWG, FCR carcass properties and abdominal fat content at 28 and 42 days compared to transport stress for 0.75 or 3 hours with no CrM. Also, discrepant outcomes from that indicated by Al-Tamimi et al. (2019), that weekly BW, BWG, FI, FCR carcass yield did not change at 5 weeks in acute heat stressed broilers for 3 hours daily which fed 1.2 g CrM /kg alone or its mixture with 1 g betaine/kg although CrM mitigated hyperthermia responses and improved the survivability. Our results are in line with those stated by Sarica et al. (2007), that 50 mg/kg Car added to diet containing sunflower or fish oil as fat source with 12.13 or 11.72 MJ/kg of metabolizable energy did not affect BW, BWG, FCR and relative weights of breast, thigh drumstick, heart and gizzard of quails up to 35 days. Similarly, it was observed that 150 mg/kg Car had no influence on final BW, BWG, FCR, carcass yield and percentages of breast, wing and heart at 42 day of quails (Tufan et al., 2015). Improved survivability in stressed birds (T3,T4 and T5) might belong to the powerful activity of CrM to support the immune system by stimulatory influence of the macrophages, monocytes and other functional immune cells which could prevent multiple diseases, inflammatory reactions, tumorigenesis and microbial infection (Lawler et al., 2002; Wu, 2020). Besides, many results revealed that dietary Car reduced the mortality by its motivation the immune system through elevated serum lysozyme, immunoglobulin-G and bactericidal activity with high lymphocytes counts in spleen of Japanese quails (Mahmoud et al., 2020) and ameliorative antibody titers against avian influenza and Newcastle disease viruses in broiler (Azizi-Chekosari et al., 2021) and Japanese quails (Abdel-Fattah et al., 2014).

A notable improvement in PER and EER at 1-3 weeks for T4 and T5 might be related to the superior apparent nutrients digestibility (Table 4), increased numbers of lactic acid in T3 and T5 and reduced numbers of total coliform and *E.coli* in T4 and T5 compared to T2 (Table 5). It is undeniable that enhanced microbiota community in avian gut has ability to resist injurious and non-injurious stressors, exclusion of pathogens, maintaining normal intestinal function, influential impacts of endogenous secretions, nutrient metabolism and digestion process (Shang et al., 2018). These data are in disagreement with

Murali et al. (2015) who conveyed that was no influence of 900 mg/kg Car in diet contained 5% animal fat on nutrient digestibility of dry matter, crude protein, nitrogen free extract, ether extract at 6 weeks old of broilers. Also, this was different from what obtained by Azizi-Chekosari et al. (2021) who found low counts of *E.coli* and coliform and high counts of lactobacilli in cecum of broilers through addition 400 mg/kg Car compared to control at 42 days.

From present results of stress indicators shown in Table 6 there were enhanced GPx, SOD, CAT, GSH, and FRAP levels and lowered LOOH, MDA, cortisol levels for quails in T5 and T4 followed by T3 in comparison to T2 which might interpret to a great extent the improved results in productive performance, ileal microbiome and nutrients digestibility. Similarly, growing body of evidence referred that was a reduction in blood antioxidant potential by reduction of antioxidative enzymes (SOD, CAT, GPx) and FRAP with increased levels of lipid peroxidations markers (MDA, LOOH) in stressed birds by lead acetate (Al-Shammari, 2017; Al-Shammari and Batkowska, 2021) or H_2O_2 (Al-Shammari et al., 2019a; Al-Shammari et al., 2019b). Impaired redox status and negative stress markers in T2 could be linked to detrimental impact of lead to increase expression levels of caspase-3 and caspase-9 (apoptotic proteins) *in vivo* that responsible for apoptosis and breaking down functional proteins in hepatic tissue of quails (Arslan et al., 2022) and this could explain in turn the impairment in blood biochemical profile in T2 (Table 7). Also, it is well know the stress could inhibit cytoprotective function of antioxidant system in broilers which is probably related to enhanced heat shock protein 70, alpha-1-acid glycoprotein, serum amyloid A and serum cortisol levels. Cortisol is the principle hormone that used as measurable biomarkers of stressful and inflammatory conditions or organ injury in poultry species (Alghirani et al., 2023). Cr might act potential mechanisms to support of antioxidant activity and scavenge the harmful free radicals such as peroxynitrite and peroxide anions in brain and skeletal muscles (Lawler et al., 2002) that are generally concerned with apoptosis, mitochondrial disorders and ATP depletion which cause myocardial infarction and neurological injuries (Wu, 2020). In broiler chickens, it was confirmed by Ibrahim et al. (2015) that was obvious reduction in MDA and increase in GSH through mixed addition at 0.05% each of CrM and whey protein concentrate. Conversely, in transport-stressed male broilers for 3 hours during summer, was shown that dietary 600 or 1200 mg/kg CrM had no influence on antioxidant capacity by no changing in in SOD, CAT and GPx activity measured in the muscles (Wang et al., 2015). Moreover, the improvement in certain antioxidant system in Car individually (T3) or with CrM (T5) may due to the importance of Car additive as antioxidant material by different ways such as immediate elimination of harmful free radicals, generation of antioxidant enzymes (GPx, glutathione reductase, glutathione transferase, CAT and SOD) and binding of stimulating metals (Cu and Fe) for reactive oxygen species synthesis (Adabi et al., 2011). Also, it was confirmed that suppression of enzymes (NAPDH oxidases and xanthine oxidase) which generate ROS, pivotal effect of redox signaling proteins (Nrf2, PPARα and NF-κB) and modulatory synthesis of thioredoxins, heat shock proteins, sirtuins and vitagenes are strictly involved in Car antioxidant potential (Surai, 2015). All these beneficial influence of Car may provide protection from ROS attack

and toxic effects and maintain mitochondrial integrity in poultry (Rehman et al., 2017). These results were in agreement nearly with reports of Wang et al. (2013) and Yousefi et al. (2013) who declared that dietary Car at 100 mg/kg increased level of SOD and GPx in heart tissue of broilers at 21, 35 and 42 days exposed to low-temperature and hypobaric hypoxia stress, respectively. The same results were documented regarding Car activity for increasing CAT in muscles and liver (Mahmoud et al., 2020) of quails with no changing in plasma corticosteron level (Zhang et al., 2014) of broiler chickens. However, differently from us, a lowering of MDA amount in serum (Abdel-Fattah et al., 2014) and edible meat (Sarica et al., 2007) of quails fed Car was observed.

The overall low enzymatic activity (AST, ALT, ALP, GGT and CK) for T5, T4 and then T3 (Table 7) is probably the reason of improved antioxidant capacity pool in these groups (Table 5). Similar results were noted that impaired antioxidant system in stressed poultry was accompanied with significant high levels of plasma creatinine, AST and ALT (Al-Shammari et al., 2019b; Arslan et al., 2022). Low creatinine or unchangeable glucose levels in T5 could confirm the importance of CarCrM to promote kidney health or reduce gluconeogenesis pathways and thus maintain the availability of glucose synthesis under stressful environment. The present result was in accordance with investigations of Chen et al. (2011) who demonstrated that no significant influence in serum creatinine and hepatic ALT with low hepatic AST at 42 days of broiler received 3% Cr in diet. Lowering in AST and ALP enzymes in T4 was probably to key role of CrM in alleviating stress. Obviously, hepatic enzymes are overproduced in case of damaged hepatocytes under stress and thus leading to increasing their high levels into blood stream (Surai, 2016). On the other hand, no changed CK activity in T4 compared with T2 is probably due to its normal function of CrM to counteract stress and fulfill high-energy demands in muscle metabolism to maintain cellular ATP and reversible transfer of the γ-phosphate group of ATP to the guanidino group of Cr to produce ADP and creatine phosphate (Wyss and Kaddurah-Daouk, 2000). Moreover, Car protects liver and mitochondria from toxic products and inflammation by enhanced liver function (Arslan, 2006; Rehman et al., 2017) and thus was clearly in reduction of hepatic enzymes (ALP and CK) in current data. Different results were reported that 100 mg/kg Car added to diet had no effect on ALP and CK activity although no changing was also found in serum ALT and AST (Yalçin et al., 2005) of laying quails.

CONCLUSIONS

Diet supplemented with combination of 250 mg/kg each of Car and CrM (CarCrM) seems to have equivalent effect to single use of 500 mg/kg CrM with respect to ameliorative blood antioxidant activity network in stressed quails by 2.5 ppm LA in drinking water. However, individual use of 500 mg/kg Car appeared a slight or unchanged effect in general results. Thus, diets supported by these additives were accumulatively efficient in improving overall productive performance, apparent digestibility, ileal eubiosis and certain blood biochemical indices of birds until 6 weeks of age.

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AUTHOR CONTRIBUTIONS

Karrar Imad Abdulsahib Al-Shammari prepared the data, carried out the analysis and wrote the manuscript and has read and approved the final manuscript before submission to the journal.

CONFLICT OF INTEREST

The author declare no conflicts of interests.

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