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Effects of *in ovo* injection of microalgae on hatchability, antioxidant and immunity-related genes expression, and post-hatch performance in broilers and Japanese quails

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ABSTRACT

The effects of in ovo injection of Arthrospira (Spirulina) Platensis on hatchability, antioxidant and immunity-related genes expression, and hatchling performance of broilers and Japanese quails was assessed in 2 separate experiments. In experiment 1, a total of 240 fertilised quail eggs were divided into six groups: control (without injection), sham control (0.02 CC distilled water/ egg), 0.75 mg Spirulina Platensis (SP)/egg, 1.5 mg SP/egg, 2.5 mg SP/egg, 3.5 mg SP/egg. In experiment 2, a total of 192 fertilised broiler breeder were divided into four groups: control, sham control (0.2 CC distilled water/egg), 25 mg SP/egg, 35 mg SP/egg. In experiment 1, in ovo injection of SP (1.5–3.5 mg/egg) increased hatchability of chicks (p < .01). In the groups received 2.5 and 3.5 mg SP/egg the expression of HSP70 and GPx genes was lower and higher than control groups, respectively (p < .001). The expression of interferon-gamma (IFN- γ) in the chicks consumed 1.5–3.5 mg SP/egg was higher than control groups (p < .001). The groups received 1.5–3.5 mg SP had lower feed conversion ratio (FCR, p < .05). Catalase activity in hatchlings consumed 2.5 or 3.5 mg SP was the highest (p < .05). In experiment 2, in ovo injection of 25 or 35 mg SP caused the lowest HSP70 in chicks (p < .0001). During the whole period of rearing, feed intake (FI) and FCR of the broilers in ovo fed with 35 mg SP was higher than control groups (p < .01). SP can be considered as an organic supplement in hatcheries for improving hatchability and anti-oxidant status of guail and broiler hatchlings.

HIGHLIGHTS

- Spirulina platensis have the potential to be considered as a functional organic supplement in commercial hatcheries.
- In ovo injection of Spirulina platensis can improve hatchability, antioxidant, and immunityrelated gene expression in quail chicks.
- In ovo injection of Spirulina platensis has positive effect on heat tolerance of broiler embryo during last days of incubation.

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Introduction

Heat stress is one of the critical events that has a detrimental effect on poultry welfare and performance due to oxidative damage of cells, endocrine disturbance, immunosuppression, and gut disorders (Farag and Alagawany 2018; Song et al. 2018). Chicken embryos are susceptible to oxidative stress due to high content of polyunsaturated fatty acids in their tissues (Surai et al. 1996). Moreover, it was found that up to 3% of oxygen in electron transport chain can convert to reactive oxygen molecules that can destroy DNA, proteins, and lipids (Surai 2018). Also, phagocyte cells produce reactive radicals which can injure body organs while escaping phagosome (Surai, 2018). Also, due to the high rate of body metabolism during the last days of incubation, chick embryo may be faced with heat stress. Also, the level of poly-unsaturated fatty acids in cell membranes of the embryos is high, and they are susceptible to oxidative damage because of the invasion of reactive free radicals (Selim et al.

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2012). Hatchlings have innate antioxidant defense system which contains superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), vitamin C, vitamin E, and a-lipoic acid (Ozkan et al. 2005). However, since the birds' eggs have closed environment, it seems that in ovo injection of organic antioxidant substances is one of the best ways to decrease the stress and mortality of embryos during the late phase of incubation, in addition to promote their immunity system (Foye et al. 2006). According to Uni and Ferket (2004), in ovo injection of nutrients can improve the biochemical and physiological state of an embryo. Surai et al. (2003) had shown that a balanced antioxidant system during the hatch is a critical factor for hatchlings viability. Hajati et al. (2014) reported that in ovo injection of 4.5 mg grape seed hydro-alcoholic extract/egg, as a herbal extract with high antioxidant activity, on 18th d of incubation increased hatchability of Cobb-500 broiler chickens. Arthrospira (Spirulina) Platensis microalgae include different antioxidant phytochemicals such as phycobiliproteins, phycocyanin, and allophycocyanin with the ability of radical scavenging. It was reported that phycocyanobilin of Spirulina could prevent NADPH oxidase activity and improve the antioxidant defense system by increasing glutathione and antioxidant enzyme synthesis (Hayashi et al. 2009). Also, Spirulina contains carotenoids such as β -carotene that can quench singlet oxygen damage (Tinkler et al. 1994). The other antioxidant substances in Spirulina include phenolic compounds like salicylic, trans-cinnamic, synaptic, chlorogenic, quinic, and caffeic acids (Miranda et al. 1998). Park et al. (2018) have found that adding Spirulina improved superoxide dismutase and glutathione peroxidase activities in broilers' blood due to phycocyanin, β -carotene, and phenolic compounds in the microalgae. Hajati et al. (2020) have found that SP supplementation decreased malondialdehide (MDA) level in blood of quails under heat stress. Also, it was reported that SP improved immune response of broiler chicken suffered heat stress condition (Mirzaie et al. 2018). Heat shock proteins exist in all cells, and the rate of their expression in liver is high, specially when the birds exposed to stressors (Kang et al. 2019). Hajati et al. (2020) reported that there was no difference in HSP70 gene expression in liver or heart of quails fed with SP supplement under heat stress. Researchers have found that Spirulina induced secretion of IFN- γ , as a cell-mediated immunity rpomoter, about 13.6 times more than basal levels in blood of human (Mao et al. 2000). Regards to the fact that there is not enough data about the effects of in ovo injection of SP in poultry eggs, this study were done to evaluate the effects of *in ovo* injection of *SP* on hatchability, antioxidant and immunity-related genes expression, and hatchling performance in broilers and Japanese quails.

Materials and methods

Spirulina preparation

SP algae were cultivated in modified Zarrouk's medium in August, 2019. Algae were incubated in 150-liter plastic tubs at mean temperature and irradiance of 31 ± 1 °C, 3000 lux, respectively. Harvesting was done after 14 days. After harvesting, microalgae was air-dried, ground, and analysed by AOAC (2005) procedures. The ash (942.05), crude protein (984.13), crude fat (920.39), and total phenol content (De Assis et al. 2014) of *SP* was determined as 12.42 ± 0.52 , 62.57 ± 0.35 , 4.89 ± 0.18 , 10.41 ± 0.05 mg GAE/g *SP*, respectively.

Experiment one

In ovo injection

In experiment 1, a total of 240 fertilised eggs (average weight = 12.57 g, laid by 10-wk-old breeder flock of *Japanese* quails) were divided into six groups: Control (without injection), Sham control (0.02 CC distilled water), 0.75 mg *SP*, 1.5 mg *SP*, 2.5 mg *SP*, 3.5 mg *SP*. The injection was done on 15 d of incubation into the centre of air sac of the fertile eggs after disinfection with 70% ethyl alcohol. Injection was done by 26 gauge syringe (1.00 ml volume). Then, sealed with sterile paraffin and took back to the incubator. The temperature and relative humidity of the incubator was set at 37.8 °C, 55% during the first 15 days of incubation; and 37.5 °C, 70% for the last three days.

Hatchability and hatchling weight

Hatchability of fertile eggs was calculated as below:

Hatchability %=Number of hatchlings/Number of fertilised eggs \times 100

On d 21 of incubation, after dryness of fluffs, the hatchling of each replicate were taken out of the incubator and weighed.

Growth performance of hatchlings

In experiment 1, after weighting the hatchlings, quail chicks were grown in 24 cages with dimensions of $40 \times 90 \times 20$ cm. Eight quail chicks were raised in each cage for one week. The experimental diet was formulated by WUFFDA software according to Ton et al. (2013) and it is shown in Table 1. The house

temperature, and relative humidity was kept around $35 \,^{\circ}$ C, and 65%, respectively. The house lighting program was 23 hours light, and 1 hour dark. The mean FI, BWG, and FCR of the quails in each cage was calculated as daily.

Liver anti-oxidant status

On hatch day of experiment 1, the level of MDA and catalase activity in the liver of 12 quail chicks per treatment was measured on hatch day. Briefly, one gram of liver was washed on ice with potassium phosphate buffer. MDA level was assessed as a biomarker of lipid oxidation in liver of quails hatchling by using spectrophotometric procedure according to Botsoglou

 Table 1. Ingredients and nutrient composition of the quails experimental diet in trial 1.

Ingredient (%)	1–7 d
Corn, ground	47.72
Soybean meal	37.98
Corn gluten	7.13
Soybean oil	3.00
Dicalcium phosphate	1.39
Oyster shell	0.59
Common salt	0.36
Premix ^a	0.30
DL-methionine	0.49
L-lysine hydrochloride	0.89
L-theronine	0.15
Calculated composition	
Metabolizable energy (kcal/kg)	2990
Crude protein (%)	23.50
Calcium (%)	0.65
Available phosphorus (%)	0.41
Lysine (%)	1.88
Methionine + Cysteine (%)	1.32
Threonine (%)	1.08
^a Vitamin/mineral supplementation (minim	num levels per

kg of premix); vit. A – 4,500,000 U; vit. D3 – 1,250,000 U; vit. E – 4,000 mg; vit. B1 – 278 mg; vit. B2 – 2,000 mg; vit. B6 –525 mg; vit. B12 – 5,000 mcg; vit. K3 – 1,007 mg; calcium pantothenate – 4,000 mg; niacin – 10,000 mg; choline – 140,000 mg; antioxidant – 5,000 mg; zinc – 31,500 mg; iron – 24,500 mg; manganese – 38,750 mg; copper – 7,656 mg; cobalt – 100 mg; iodine – 484 mg; selenium – 127 mg. et al. (2002). The MDA level of the liver samples was evaluated by spectrophotometry (Shimadzu, Model AA-670, Tokyo, Japan) at 530 nm wavelength, and expressed as μ g/g liver. Also, catalase activity in liver samples of quail chicks was assessed according to Aebi (1984) and expressed as units/mg protein liver.

Gene expression by real-time PCR

The expression of HSP70, GPx, and IFN-γ was assessed in liver of 1 d-old quail hatchlings in experiment 1. The glyceraldehyde 3 phosphate dehydrogenase (GAPDH), b-actin, and 18s rRNA was used for data normalisation. Briefly, on hatch day, liver of three quail chicks in each replicate was separated, put in the cryogenic tubes, and frozen in liquid nitrogen. Trizol (GeneAll, South Korea) was used to prepare total RNA. After treating samples with DNase I (Fermentase, USA), purity of extracted RNA was assessed by Nano-drop 2000 pectrophotometer (Thermo Scientific, USA). Then, about two micrograms of extracted RNA was used to obtain cDNA of the samples by using gene PAK RT universal kit (Fermentas, USA). The sequence of the primers of GAPDH (housekeeping gene), HSP70, GPx, and IFN- γ was taken from the NCBI database (Table 2). Real Time PCR 7500 Fast System (Applied Biosystems, USA) was used to amplify the genes in triplicate with the following program: 95 °C for 30 s, 40 cycles 95 °C for 15 s, 52 °C for 20 s, 72 °C for 20 s. For evaluating the gene relative expression, the $\Delta\Delta$ Ct model was used in present study, and the Ct of target gene and reference gene is considered. In order to calculate ΔCt for each target gene, the Ct number of target gene was subtracted from the Ct of housekeeping gene for each sample. $\Delta\Delta$ Ct of each gene was considered by subtracting the ΔCt of target from ΔCt of control sample. Data of gene expression were analysed by Excel software.

Table 2. Gene forward (F) and reverse (R) primer sequences in experiment	1 and experiment 2.
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Gene	Primer sequences	Tm Ref		Amplicon	
Experiment 1 (Quail)					
GAPDH ^a	F: CTTTGGCATTGTGGAGGGTC	60	NM_204305	128	
	R: ACGCTGGGATGATGTTCTGG	60			
HSP70 ^b	F: CGTCAGTGCTGTGGACAAGAGTA	60	NM_001323198	145	
	R: CCTATCTCTGTTGGCTTCATCCT	60			
IFN-γc	F: CAACTTGTTTGTTCTGTCTGTCATC	60	NM_205149	185	
	R: TTCTCATTTCTCTCTGTCCAGTTCT	60			
GPx ^d	F: CGAAAAGTGCGAGGTGAACG	60	AB371709.1	112	
	R: GTACTGCGGGTTGGTCATCA	60			
Experiment 2 (broiler)					
GAPDH ^a	F: CTGCCCAGAACATCATCCCA	60	NM_204305.1	137	
	R: GCAGGTCAGGTCAACAACAGAGAC	60			
HSP70 ^b	F: ATCAAGCGTAACACCACCATTCC	60	GU980869.1	120	
	R: GTTGTCCTTTGTCATAGCCCTCTC	60			

^aGAPDH: glyceraldehyde-3-phosphate dehydrogenase; ^bHSP70: heat shock protein 70; ^c*IFN-*₇: Interferon gamma; ^dGPx: glutathione peroxidase. Tm: melting temperatures; Ref: Reference genes bank accession number.

Experiment two

In ovo injection

In experiment 2, a total of 192 fertilised broiler breeder eggs (average egg weight = 64.24 g, from Ross 308 strain flock at 18 weeks of production) were divided into four groups: Control (without injection), Sham control (0.2 CC distilled water), 25 mg *SP*, 35 mg *SP*. Injection was done by using 24 gauge syringe on 18 d of incubation into the centre of air sac of the fertile eggs after disinfection with 70% ethyl alcohol. Then, sealed with nail polish and took back to the incubator. The temperature and relative humidity of the incubator was set at 37.5 °C, 60% during the first 18 days of incubation; and 37 °C, 75% for the last three days. Hatchability of fertile eggs was calculated as Experiment 1.

Growth performance of hatchlings

In experiment 2, broiler chicks were raised in 16 floor pens with wood litter with dimensions of $100 \times 100 \times 60$ cm. Eight broiler chicks were allocated to each pen for four weeks. The experimental diets (Table 3) were prepared according to Ross 308 nutritional guideline (2019). The initial temperature, and relative humidity of broilers' house was 32 °C, and 65%, respectively. Lighting program of the house was 23 hours light, and 1 hour dark. Growth performance traits such as BW, BWG, FI was calculated for the

Table 3. Ingredients and nutrient composition of broilersexperimental diets in trial 2.

Ingredient (%)	1–10 d	11–24 d	25–28 d
Corn, ground	51.02	54.72	55.53
Soybean meal	36.76	32.71	35.42
Corn gluten	5.00	5.00	0.00
Soybean oil	3.06	3.81	5.61
Dicalcium phosphate	1.78	1.56	1.39
Oyster shell	1.22	1.11	1.03
Common salt	0.34	0.36	0.36
Minerals mix ^a	0.25	0.25	0.25
Vitamins mix ^b	0.25	0.25	0.25
DL-methionine	0.17	0.13	0.16
L-lysine hydrochloride	0.14	0.10	0.00
Calculated composition			
Metabolizable energy (kcal/kg)	3000	3100	3160
Crude protein (%)	23.00	21.50	19.75
Calcium (%)	0.97	0.87	0.81
Available phosphorus (%)	0.48	0.43	0.40
Lysine (%)	1.28	1.15	1.10
Methionine + Cysteine (%)	0.95	0.87	0.81
Threonine (%)	0.88	0.82	0.77

^aMineral mix supplied the following per kg of diet: Cu, 20 mg; Fe, 100 mg; Mn, 100 mg; Se, 0.4; Zn, 169.4 mg.

^bVitamins mix supplied the following per kg of diet: vitamin A – 18,000 U; vitamin D3 – 4,000 U; vitamin E – 36 mg; vitamin K₃ – 4 mg; vitamin B₁₂ – 0.03 mg; thiamine – 1.8 mg; riboflavin – 13.2 mg; pyridoxine – 6 mg; niacin – 60 mg; calcium pantothenate – 20 mg; folic acid – 2 mg; biotin – 0.2 mg; choline chloride –500 mg.

chicks in each pen, daily. Also, FCR of the birds in each experimental unite was adjusted considering mortality.

Gene expression by real-time PCR

In experiment 2, HSP70 gene expression was evaluated in the liver of 1 d-old broiler hatchlings. The glyceraldehyde 3 phosphate dehydrogenase (GAPDH), b-actin, and 18 s rRNA was used for data normalisation. Briefly, on hatch day of both experiments, the liver of three broiler chicks in each replicate was separated, put in the cryogenic tubes, and frozen in liquid nitrogen. Trizol (GeneAll, South Korea) was used to prepare total RNA. After treating samples with DNase I (Fermentase, USA), purity of extracted RNA was assessed by Nano-drop 2000 pectrophotometer (Thermo Scientific, USA). Then, about two micrograms of extracted RNA was used to obtain cDNA of the samples by using gene PAK RT universal kit (Fermentas, USA). The sequence of the primers of GAPDH (housekeeping gene) and HSP70 was taken from the NCBI database (Table 2). The HSP70 gene relative expression to the GAPDH (housekeeping gene) in liver of 1 d-old broilers was assessed according to our recent study (Zaghari et al. 2020).

Statistical analysis

In order to analyse, arcsine trans-formation was used for the percentage data. The statistical model in the two experiments was a completely randomised design, and all the data was analysed by GLM procedure of SAS software (version 9.3). Duncan's new multiple range test was used to compare means of the considered traits. The level of significance was considered as p < .05.

Results

Experiment 1

Regards to Table 4, *in ovo* injection of *SP* (1.5, 2.5, 3.5 mg/egg) increased hatchability of quail chicks (p < .001). The hatchability of control, sham control, and 0.75 mg *SP*/egg groups was not different. The birth weight of quails increased by *in ovo* injection of different levels of *SP* (p < .001). However, injection of distilled water had no significant effect of the chicks' body weight compare to control group (8.81 vs. 8.61).

As data shown, the yolk residue in quail chicks which consumed 2.5 or 3.5 mg *SP* was lower than control groups on 2 or 5 d after hatch (p < .05). On d 2,

Table 4. Effects of *in ovo* feeding of *Spirulina Platensis* in *Japanese* quails on hatchability, birth weight, and performance during first week of age.

	Control		SP (mg/e.g., g)					
		Sham Control	0.75	1.5	2.5	3.5	SEM	p Value
Hatchability (%)	90 ^b	90 ^b	90 ^b	100 ^a	100 ^a	100 ^a	0.125	P < .001
Birth weight (g)	8.660 ^d	8.810 ^{cd}	8.970 ^c	9.300 ^b	9.410 ^{ab}	9.620 ^a	0.189	P < .001
Body weight at d 2 (g)	11.200 ^b	11.430 ^{ab}	11.560 ^{ab}	11.980 ^a	12.020 ^a	12.100 ^a	0.107	.263
Yolk residue % (2 d)	2.186 ^ª	2.207 ^a	2.182 ^a	1.857 ^{ab}	1.496 ^b	1.321 ^b	0.097	.239
Body weight at d 5 (g)	18.080	17.480	17.450	19.150	19.220	19.300	0.738	.205
Yolk residue % (5 d)	0.167 ^a	0.173 ^a	0.169ª	0.077 ^{ab}	0.050 ^b	0.046 ^b	0.040	.014
Body weight at d 7 (g)	25.390 ^{abc}	22.440 ^c	23.670 ^{bc}	26.140 ^{ab}	26.400 ^{ab}	27.730 ^a	1.291	.023
FI (g/bird/d)	5.490 ^a	4.880 ^b	5.170 ^{ab}	5.150 ^{ab}	5.290 ^{ab}	5.550 ^a	0.054	.016
FCR (g/g)	2.300 ^b	2.510 ^ª	2.462 ^{ab}	2.141 ^c	2.181 ^c	2.147 ^c	0.109	.049
Liver MDA (µg/g liver)	0.061	0.058	0.050	0.034	0.044	0.041	0.002	.972
Liver catalase (U/g prot)	21.215 ^b	19.775 ^b	20.148 ^b	21.788 ^b	27.345 ^a	30.389 ^a	0.582	.049

Means within the same row with uncommon superscript differ significantly (p < .05). FI: feed intake; FCR: feed conversion ratio; MDA: malondialdehyde; SP: Spirulina Platensis.



Figure 1. Effects of *in ovo* feeding of *SP* on HSP70, GPx, *IFN-γ* gene expression in liver of 1 d-old quails chicks. SP: *Spirulina Platensis*.

the body weight of guails which in ovo fed with 2.5 or 3.5 mg SP was higher than the control groups (p < .05), but there was no difference among the chicks' body weight at d 5 (p>.05). On d 7, body weight of quail chicks which in ovo fed with distilled water (Sham control) was lower than guails consumed 1.5, 2.5, or 3.5 mg SP/egg (p < .05). During the first week of rearing, the FI of sham control group was lower than the control group, and this group had higher FCR (p < .05). The groups consumed 1.5, 2.5 or 3.5 mg SP had lower FCR compared to other groups (p < .05). Moreover, catalase activity in liver of quail hatchlings consumed 2.5 or 3.5 mg SP was higher (p < .05). There was no significant difference in the level of liver MDA (p>.05), however, in ovo injection of different levels of SP decreased MDA level compared to control groups numerically.

As Figure 1 shows, gene expression of HSP70 in liver of day-old quail chicks consumed 2.5 or 3.5 mg *SP*/egg was lower than the control and sham control groups (0.742 or 0.671 vs. 1 &0.969, p < .001).

Also, gene expression of GPx in liver of day-old chicks received 2.5 or 3.5 mg SP/egg was higher than

the control groups (1.539 or 1.973 vs. 1 & 0.913, p < .001). There was no difference in HSP70 and GPx gene expression among the control, sham control, 0.75 mg *SP*/egg, and 1.5 mg *SP*/egg groups. Besides, IFN- γ gene expression in the liver of groups fed 1.5, 2.5, or 3.5 mg *SP*/egg were higher than the control groups (p < .001). However, IFN- γ gene expression in the liver of chicks in control, sham control, and 0.75 mg *SP*/egg was not significantly different.

Experiment 2

In ovo injection of the different levels of *SP* increased hatchability of the broiler chicks numerically (Figure 2). Also, there was no difference among the body weight of chicks on hatch day (p>.05). Regards to Table 5, hatchlings yolk residue and relative liver weight to live body weight in the sham control group was higher and lower than control group, respectively (p < .05). As data have shown, the relative heart weight of hatchlings in different groups was not different (p>.05).



Figure 2. Effects of in ovo feeding of SP (25, 35 mg/egg) on hatchability of broiler chicks SP: Spirulina Platensis.

Table 5. Effects of *in ovo* feeding of *SP* in broiler chicks on birth weight, relative heart and liver weight (%), and yolk sac residue (%) on day of hatch. SP: *Spirulina Platensis*.

			<i>SP</i> (mg/e.g., g)			
	Control	Distilled water	25	35	SEM	p Value
Birth weight (g)	47.760	49.850	49.760	48.580	0.633	0.618
Heart (%)*	0.883	0.814	0.854	0.889	0.021	0.544
Liver (%)*	2.478 ^a	1.968 ^b	2.440 ^a	2.611ª	0.077	0.006
Yolk residue (%)*	14.430 ^{ab}	18.540 ^a	10.970 ^b	10.640 ^b	1.071	0.013

Means within the same row with uncommon superscript differ significantly (p < .05).*Organweight/Liveweight*100.



Figure 3. Effects of *in ovo* feeding of SP (25, 35 mg/egg) on HSP70gene expression in liver of 1 d-old broiler chicks. SP: Spirulina Platensis.

Figure 3 illustrated the effects of *in ovo* injection of *SP* on the liver HSP70 gene expression of 1 day-old chicks. The HSP70 gene was expressed less in broiler chicks which consumed 25 or 35 mg *SP* on d 18 of incubation compared to the control groups (0.466 or 0.407 vs. 1 & 0.801, p < .0001).

As Table 6 shows, there was no significant difference among growth performance traits of broilers during the first and third weeks of the experiment (p>.05). The FI of the broiler chicks received 35 mg *SP*/ egg was higher than the sham control group during the second week of the experiment (p < .05). During the fourth week of the experiment, groups received 25 or 35 mg *SP*/egg had higher FI than control groups (p < .001). Also, FCR of the group received 35 mg *SP*/

egg was higher than control groups during the fourth week (p < .01). FI and FCR of the chicks consumed 35 mg *SP* on d 18 of incubation was higher than control groups during the whole period of the experiment (p < .01). Also, the BWG of sham control group was lower than control group during 1–28 d of age (p < .05).

Discussion

It is well known that birds have two main procedures against oxidative stress. First, enzymatic protection by endogenous antioxidant materials such as catalase, glutathione peroxidase, superoxide dismutase, etc., which can eliminate reactive oxygen or nitrogen

	Control Distilled water		<i>SP</i> (mg/e.g., g)			
		25	35	SEM	$\Pr > F$	
D1-7						
FI (g/d)	30.086	29.564	30.052	30.532	0.239	.089
BWG (g/d)	29.773	29.950	30.077	29.862	0.282	.886
FCR (g/g)	1.010	0.987	0.999	1.022	0.011	.198
D 8–14						
FI (g/d)	73.885 ^{ab}	73.375 ^b	74.422 ^{ab}	74.862 ^a	0.325	.036
BWG (g/d)	53.631	52.629	53.546	53.395	0.303	.131
FCR (a/a)	1.377	1.394	1.389	1.402	0.011	.524
D15-21						
FI (g/d)	109.500	108.857	109.914	110.678	0.570	.202
BWG (g/d)	80.852	80.008	80.179	79.954	0.464	.520
FCR (a/a)	1.354	1.360	1.370	1.384	0.009	.212
D21-28						
FI (a/d)	164.286 ^c	163.500 ^c	165.179 ^b	166.107 ^a	0.271	p<.001
BWG (a/d)	109.161	108.371	108.528	108.071	0.297	.120
FCR (a/a)	1.505 ^b	1.508 ^b	1.522 ^{ab}	1.537 ^a	0.005	.005
Total (D 1–28)						
FI (g/d)	377.760 ^b	375.296 ^c	379.569 ^b	382.182 ^a	0.792	p<.001
BWG (g/d)	247.940 ^a	245.232 ^b	246.546 ^{ab}	247.943 ^a	0.559	.026
FCR (g/g)	1.523 ^b	1.530 ^b	1.539 ^{ab}	1.554 ^a	0.005	.009

Table 6. Effects of *in ovo* feeding of SP in broiler chickens on the performance of the broiler chicks up to fourth week of age. SP: Spirulina Platensis.

Means within the same row with uncommon superscript differ significantly (p < 0.05). FI = feed intake; BWG = body weight gain; FCR = feed conversion ratio.

species and help to transform them into less active substances (Surai 2000; Halliwell and Gutteridge 2015). The second defense mechanism is through nonenzymatic antioxidant materials such as vitamin E, vitamin C, polyphenols, etc., which help antioxidant enzymes as cofactors. Many of such antioxidant substances cannot be synthesised in birds body or not provided inadequate amount, so they should be provided in feed before and after hatching of eggs (Møller et al. 2000). During last days of incubation, birds embryos may be suffered from heat stress due to high metabolic rate and hot environment of incubator (Tullett 1990). After internal pipping, pulmonary respiration begins and the occurence of oxidative stress weakness of innate immunity system is more probable (Moran 2007; Yigit et al. 2014). Oxidative stress can reduce liveability of embryos; thus it can decrease the hatchability of fertile eggs. In present study, in ovo administration of SP improved hatchability and birth weight of quails. Moreover, SP improved hatchability and birth weight of broiler chicks numerically. It is well known that SP has antioxidant activity that relates to its phenolic compounds and natural pigments like: β -carotene, β -cryptoxanthin, chlorophyll, echinenone, myxoxanthophyll, phycoerythrin, phycocyanin, xanthophylls, and zeaxanthin (Gad et al. 2011; Zaid et al. 2015; Asghari et al. 2016; Hajati and Zaghari 2019). In present study, total phenol and selenium content of SP as antioxidant substances assessed in this study was about 10.41 ± 0.05 mg GAE/g and 20.00 mcg/100 g, respectively. It seems that antioxidant

activity of SP could improve oxidative state of embryos during late phase of incubation, so more chicks which in ovo fed with SP could hatch. Selenium content of Spirulina microalgae is in the forms of Selenomethionine and Selenocysteine and other derivatives (Cases et al. 2001). In a recent study, Surai and Kochish (2019) had found that providing adequate dietary levels of selenium, vitamin E, carotenoids, and other antioxidants is necessary for poultry suffering stress situation. Selenium has positive effect on birds antioxidant system through non-enzymatic (GSH, CoQ, vitamin E) and enzymatic (superoxide dismutase) processes. In poultry nutrition, it should be considered that form (organic/inorganic) and dietary level of trace elements such as selenium affect their antioxidant efficiency in birds body. It is well documented that organic form of trace elements are more efficient than inorganic forms (Surai and Kochish 2019). The activity of selenium-dependent glutathione peroxidase in the liver of chicks embryo increase continuously during the second part of incubation period, and its maximum level is during hatch time to defense against hatch stress (Surai 1999). Regards to this finding, quail chicks which in ovo fed with 2.5 or 3.5 mg SP had lower yolk residue. This can be due to the microalgae' s positive effect on beneficial intestinal microbiota, absorption, and digestion processes in animals (Seyidoglu et al. 2017). In present study, in ovo injection of SP had positive effect on hatchlings birth BW and performance of the quail chicks during the first week post-hatch. SP lipids include w₃ fatty acids,

mainly linolenic acid that has the potential for converting to prostaglandin, eicosapentaenoic acid, and docosahexaenoic acid. It was proved that the consumption of these fatty acids could promote embryo's growth and prevent being sick (Swanson et al. 2012). Besides, some researchers have found that adding SP to broilers diet supported the birds growth performance (Bellof and Alarcón 2013). As mentioned above in this study, SP injection in broiler breeders' eggs at the level of 25 mg/egg had not any significant effect on hatchling growth performance, but injection of 35 SP mg/egg adversely affected broilers performance during fourth week of rearing. As we did not see any significant effect on growth traits up to third week post-hatch, the adverse effect on FCR of the broilers during fourth week of the experiment is maybe related to housing management factors. Because of a lack of information about in ovo injection of SP in birds, further study is needed to reach a comprehensive conclusion. It is well known that heat stress can cause oxidative stress lead to lipid peroxidation in muscle cells of chickens (Mujahid et al. 2007). Oxidative stress disturbs the oxidants-antioxidants balance in broilers and MDA level increases likewise activity of serum GPx decreases (Georgieva et al. 2006). Our data was shown that the activity of liver catalase in quail hatchlings in ovo fed with 2.5 or 3.5 mg SP/ egg was higher than control groups on hatch day. Hajati et al. (2020) reported that adding 0.5% SP to laying quails diet caused the lowest MDA, and heterophil to lymphocyte ratio (heat stress biomarkers) in blood of the birds.

Consistently, Park et al. (2018) have reported that adding dietary SP improved serum antioxidant enzyme activities in broiler chickens. This can relate to SP antioxidant substances such as C-phycocyanin, selenium phenolic acids, betacarotene, tocopherol, and chlorophyll with strong antioxidant properties (El-Desoky et al. 2013; Park et al. 2018). It was reported that phycocyanin's anti-oxidant property is about 16, and 20 times stronger than Trolox, and vitamin C, respectively (Romay and Gonzalez 2000). Additionally, a recent study has shown that organic selenium of dietary SP improved catalase activity in rabbits (Hassan et al. 2019). It is specified that several pathological (such as viral diseases) and physiological situations (like heat stress) can lead to heat shock protein production in animals, which have the potential of promoting immunity responses (Kregel 2002). Data of the present study have shown that in ovo injection of SP has lowered hepatic gene expression of HSP70 in guail and broiler chicks. Moreover, hepatic GPx gene expression was upregulated in guail chicks received SP. It is well known that selenium is one of the most critical trace minerals which is important for GPx activity. However, heat stress condition can decrease selenium concentration in body cells, so it also decrease GPx activity (Gan et al. 2013). In addition to determined substances of SP with antioxidant activity, it was propounded that phospholipids, amines, vitamin C, and phenolic compounds of algae can regenerate vitamin E (Le 1990). Also, SP microalgae has been considered as one the organic sources of selenium, and consistent to our result, researchers have found that adding organic selenium such as selenium yeast to birds diets decreased HSP70 expression (Mahmoud and Edens 2005; Rivera et al. 2005). In the present study, in ovo injection of 1.5, 2.5, or 3.5 mg SP/egg increased hepatic IFN- γ gene expression in newly hatched quails. IFN- γ is a cytokine that activates macrophages and improves T-helper 1 activity to support cell-mediated immune response (Boehm et al. 1997). The balance of T-helper1/T-helper 2 is crucial to recognise which process is happening at higher rate: macrophage activity or antibody secretion (Balachandran et al. 2006). In accordance with our findings, Mao et al. (2000) found that Spirulina improved the IFN- γ level in blood to support the balance of T-helper 1 and T-helper 2 production. Therefore, it seems that SP has the potential to improve immune responses that can be due to its certain nutrient content such as immulina polysaccharide, C-phycocyanin, beta-carotene, selenium, tocopherol, vitamin C, gamma-linolenic and linolenic acids (Seyidoglu et al. 2017). Also, calcium-spirulan is a sulphated polysaccharide derived from SP with antiviral activity and can improve immunity responses (Pugh et al. 2001). Contrary to our results, Seyidoglu et al. (2017) reported that using SP (5%) in rabbits' diet did not have a significant effect on cytokines IFN- γ and IL-4 expression. This diversity in researchers findings can be related to differences in animals classes, dosage of SP usage, house condition, etc.

Conclusion

In conclusion, *in ovo* injection of *SP* on day of transfer eggs from setter to hatcher had a positive effect on the hatchability, antioxidant, and immunity-related gene expression in quail chicks. Moreover, FCR of the quail chicks *in ovo* injected with *SP* at the level of 1.5–3.5 mg/egg decreased during first week posthatch. In addition, *in ovo* injection of 25 mg *SP*/egg decreased HSP70 gene expression in newly hatched broiler chicks without any adverse effect on their post-

hatch performance. So, *SP* can be considered as a functional organic supplement in commercial hatcheries to improve hatchability and the health state of hatchlings.

Ethical approval

All procedures were approved by the Institutional AnimalCare and Ethics Committee of the Iranian Council of AnimalCare (Care ICoA, 1995)

Disclosure statement

No potential conflict of interest was reported by the author(s).

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