Dietary combined supplementation of iron and *Bacillus subtilis* enhances reproductive performance, eggshell quality, nutrient digestibility, antioxidant capacity, and hematopoietic function in breeder geese

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ABSTRACT A 3 × 2 factorial arrangement of treatments was conducted to investigate the effects of iron (Fe, 40, 60, and 80 mg/kg) and *Bacillus subtilis* (2.5×10^9 and 5.0×10^9 CFU/kg) supplementation on reproductive performance, egg quality, nutrient digestibility, hormone levels, antioxidant indices, and hematological parameters in breeder geese. A total of one hundredtwenty 46-week-old Wulong breeder geese were randomly assigned to 1 of 6 dietary treatments with 4 replicates per treatment and 5 geese per replicate for 10 wk following 1 wk of adaption. Dietary Fe supplementation increased egg weight ($P = 0.036$), fertility ($P = 0.022$), serum total antioxidant capacity ($P = 0.022$), red blood cell ($P = 0.001$), hematocrit (**HCT**, $P < 0.001$), hemoglobin (**HGB**, $P = 0.005$), and mean corpuscular volume (**MCV**, $P < 0.001$). Dietary *B. subtilis* supplementation increased egg production ($P = 0.025$), eggshell thickness ($P = 0.020$), apparent phosphorus digestibility ($P < 0.001$), serum follicle stimulating hormone ($P = 0.043$), total antioxidant capacity ($P < 0.001$), HCT ($P < 0.001$), HGB ($P < 0.001$), and MCV ($P = 0.025$), and reduced malondialdehyde level ($P = 0.008$). The birds fed diets supplemented with 60 mg/kg Fe and 5×10^9 CFU/kg *B. subtilis* showed the highest percentage of hatched eggs ($P = 0.004$) and mean corpuscular hemoglobin ($P < 0.001$) among the 6 groups. Supplementation of 40 and 60 mg/kg Fe significantly increased the apparent digestibility of calcium compared with that of 80 mg/kg Fe in the birds fed 5.0×10^9 CFU/kg *B. subtilis* ($P = 0.004$). Supplementation with 60 and 80 mg/kg Fe in the birds fed 5×10^9 CFU/kg *B. subtilis* significantly decreased serum urea nitrogen level compared with other 4 groups ($P = 0.022$). In conclusion, the combination of Fe and *B. subtilis* effectively improves reproductive performance, eggshell quality, nutrient digestibility, antioxidant status, and hematopoietic function of breeder geese. Dietary addition of 60 mg/kg Fe and 5.0×10^9 CFU/kg *B. subtilis* was an optimum supplementation dose.

Key words: iron, *Bacillus subtilis*, reproductive performance, hematopoietic function, breeder goose

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INTRODUCTION

Iron (**Fe**) is an essential trace element required for a varied number of metabolic processes in poultry, including participating in oxygen and electron transport, energy supply, protein metabolism, antioxidant activity, and immunity (von Drygalski and Adamson, 2013; Abbaspour et al., 2014). Dietary Fe concentration has been reported to influence the contents of Fe in the yolk, hatchability of fertile eggs, and hematocrit and hemoglobin of hens and chicks (Morck and Austic, 1981; Paik et al., 2009; Taschetto et al., 2017). Iron supplementation also contributed to improving antioxidant capability and immunity of animals (Oppenheimer, 2001; Xie et al., 2019; Zafar and Khan, 2020). Iron deficiency may cause anemia or physiologic derangement (Baker and Greer, 2010). Iron deficiency anemia reduced animal's immunity and weight or even caused death (Godyń et al., 2016). However, long-term intake of excessive Fe would produce reactive oxygen species, which could cause pathological changes through lipid peroxidation and DNA damage (Nicholls and Budd, 2000; Zödl et al., 2003). What's more, overdose of Fe

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being excreted from organism will pollute environment and will also result in a waste of Fe. Dietary Fe requirement for breeder geese is 60 ppm, which is an estimated value based on the requirements of chickens (NRC, 1994). Limited information has been published on Fe requirement for breeder geese.

Probiotics are living microorganisms, which confer health benefits to the host when administered in sufficient amounts. *Bacillus subtilis*, a spore-forming probiotic, is metabolically dormant to face extreme environments, such as extreme high or low temperatures and pH (Nicholson, 2002). Therefore, *B. subtilis* is ideally suitable for the application in pelleted feeds. *B. subtilis* is also known to be an aerobic bacterium that consumes large amounts of free oxygen in the digestive tract when proliferating. It can inhibit the growth of aerobic pathogens and promote the proliferation of anaerobic probiotic such as *Lactobacillus* and *bifidobacterium* (Abdelqader et al., 2013; Guo et al., 2017). Moreover, *Bacillus* species produce a variety of extracellular enzymes, such as protease, cellulase, amylase, and lipase (Schallmey et al., 2004). Dietary *B. subtilis* exerts positive effects on enhancing egg production (Chen et al., 2019), feed efficiency (Yang et al., 2019), egg quality (Xu et al., 2006; Neijat et al., 2019), intestinal health (Abdelqader et al., 2013), and immune function (Zhu et al., 2019) and reducing oxidative stress (Rajput et al., 2013; Bai et al., 2016) in poultry.

Our previous results revealed that dietary *B. subtilis* and certain trace element (copper, manganese, zinc, or iron) synergistically increased the growth performance of meat geese; thus, the amount of this trace element in the diet could be reduced. However, rare studies have been conducted about the combined effects of Fe and *B. subtilis* supplementation on breeder geese. Therefore, the purpose of this study was to investigate the synergetic effects of Fe and *B. subtilis* on production and hatching performance, egg quality, nutrient digestibility, hormone levels, antioxidant indices, and hematological parameters in breeder geese.

MATERIALS AND METHODS

Animals and Experimental Treatments

This study was approved by the Animal Care and Use Committee of Qingdao Agricultural University. A total of one hundred twenty 46-wk Wulong breeder geese were obtained from the Quality Waterfowl Research Institute of Qingdao Agricultural University. Geese were allocated into 6 treatment groups, and each group consisted of 4 replicate cages. Each cage included 1 male and 4 female breeder geese, which mated naturally. The experiment was designed as a 3 × 2 factorial arrangement including 3 dietary Fe supplementation levels (40, 60, and 80 mg/kg Fe as $FeSO_4 \cdot H_2O$) and 2 dietary *B. subtilis* supplementation levels (2.5×10^9 and 5.0×10^9 CFU/kg). Initial body weights were similar across all the groups. Iron and *B. subtilis* were purchased from Qingdao Puxing Biotechnology Co., Ltd. (Qingdao, China). The basal diet was formulated according to the nutrient requirements for breeder geese as recommended by National Research Council. (1994). Diet composition and nutrient levels are shown in Table 1. All diets were in mash form. The Fe concentration of the basal diet was 71.94 mg/kg, as determined by inductively coupled plasma-atomic emission spectrometry. The experimental period lasted 10 wk following 1 wk of adaption to the dietary treatments. All geese were reared on floor and had free access to feed and water.

Production and Hatching Performance

Feed consumption, egg number, total egg weight, and number of hatched eggs per replicate were recorded daily to calculate feed intake, egg mass, feed conversion ratio, egg production, egg weight, and percentage of hatched eggs throughout the entire experimental period. Percentage of hatched eggs was defined as the percentage of hatched eggs over the number of total eggs laid throughout the entire experimental period. Only eggs that were clean and without visible abnormalities were considered as hatched eggs. In the last week of the study, 20 eggs per treatment (4 replicates per treatment and 5 eggs per replicate) were randomly selected to be incubated under standard conditions. Fertility was defined as the percentage of fertilized eggs over the number of total eggs incubated. Hatchability was defined as the percentage of goslings hatched over the number of total eggs incubated.

Egg Quality

In the last but 1 wk of the study, 12 eggs per treatment (4 replicates per treatment and 3 eggs per replicate) were randomly selected to measure egg quality within 48 h. Eggshell strength, albumen height, yolk color, and Haugh unit were measured using a digital egg tester (DET-6000, NABEL Co. Ltd., Kyoto, Japan) immediately. Eggshell thickness was determined by the average values from the 3 different locations (bottom, middle, and top of the egg) by a dial pipe gauge (Mitutoyo, Kawasaki, Japan). Egg length and egg width at midpoint on the outer surface of the egg were measured by a digital vernier caliper (0.01 mm, Mitutoyo), and egg shape index was calculated as the ratio of length to width. The yolk ratio was calculated by the formula: yolk ratio (%) = yolk weight (g)/egg weight (g) × 100%.

Sample Collection

At the end of the experiment, 8 female geese (2 geese per replicate) were randomly selected from each treatment group. Two portions of blood (5 mL each) from each goose were individually collected via wing venipuncture. One was collected into serum vacutainer tube, centrifuged at 3,000 r/min for 10 min, and stored at −20°C until the analysis of hormones, urea nitrogen, and antioxidant parameters. The other was collected

Table 1. Composition and nutrient levels of basal diet (air-dry basis).

Ingredients	Content (%)	Nutrient levels [3]	Content
Corn	59.50	Metabolizable energy (Mcal/kg)	2.73
Soybean meal	19.45	Crude protein (%)	16.27
Fish meal	3.00	Crude fiber (%)	4.03
Wheat bran	0.50	Ca (%)	2.89
Dicalcium phosphate	1.25	Nonphytate phosphorus (%)	0.47
Limestone	5.60	Lys (%)	0.85
Husk powder	7.63	Arg (%)	0.95
Soybean oil	1.90	Met + Cys (%)	0.70
Sodium chloride	0.40	Fe (mg/kg)[4]	71.94
Trace mineral premix[1]	0.50		
Vitamin premix[2]	0.11		
D,L-Methionine, 98%	0.16		
Total	100.00		

[1]Trace mineral premix provided the following (per kg of diets): Se 0.5 mg, Zn 50 mg, Mn 30 mg, Cu 4 mg, I 0.3 mg.
[2]Vitamin premix provided the following (per kg of diets): vitamin A 9,000 IU, vitamin D_3 2,000 IU, vitamin E 40 mg, vitamin K_3 0.8 mg, vitamin B_1 2.0 mg, vitamin B_2 4.0 mg, nicotinamide 30 mg, pantothenic acid 11 mg, vitamin B_6 4.0 mg, biotin 0.2 mg, folic acid 0.5 mg, vitamin B_{12} 12 μg.
[3]Calculated value except for Fe.
[4]Analyzed concentration.

into heparinized vacutainer tube to detect hematological parameters.

Serum Hormone and Urea Nitrogen Analysis

Serum follicle stimulating hormone (**FSH**), estradiol (**E2**), and prolactin (**PRL**) levels were determined with commercial radioimmunoassay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's guidelines. Serum urea nitrogen level was determined using commercial colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol.

Serum Antioxidant Parameter Measurements

The total antioxidant capacity (**T-AOC**), catalase, and total superoxide dismutase activities, and malondialdehyde (**MDA**) level were determined using colorimetric kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's instructions.

Hematological Parameter Analyses

The red blood cell (**RBC**) count, hematocrit (**HCT**), hemoglobin (**HGB**), mean corpuscular volume (**MCV**), and mean corpuscular hemoglobin (**MCH**) were determined by an automatic biochemical analyzer (7600-020, Hitachi Inc., Tokyo, Japan).

Metabolism Trial and Nutrient Digestibility Analyses

At the end of the experiment, 1 female geese per replicate was selected to feed separately for metabolism trial. The metabolism trial lasted for 8 D, including 4-D adaption period, 1-D fasted period, and subsequent 3-D collection period. Daily feed intake, residual of diets, and feces were collected, weighed, and recorded. Hydrochloric acid (10% v/v) was added at a dose of 0.1 mL/g fresh fecal to prevent nitrogen-volatilization. Samples of diets and excreta were stored at −20°C. The feed and excreta samples were oven-dried at 65°C for 72 h to constant weight, ground to pass through a 1 mm sieve, and analyzed for crude protein, ether extract, and crude fiber using the method of AOAC (AOAC 2001). Calcium (**Ca**) contents were determined by permanganate titration according to GB/T 6436-2018 (People's Republic of China National Standard, 2018), and total phosphorus (**P**) contents were assayed by phosphorus vanadium molybdate yellow colorimetric method and GB/T 6437-2002 (People's Republic of China National Standard, 2002).

Statistical Analysis

The results are presented as the mean and SEM. Data were analyzed using a 2-factor ANOVA of a general linear model in SPSS 20.0 (Chicago, IL). One-way ANOVA and Duncan's multiple comparison were used when a significant interaction was observed. When the main effect of Fe concentrations was significant, Duncan's multiple comparison were performed over the main effect means for Fe concentrations (averaged over *B. subtilis* concentrations). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Production Performance

As shown in Table 2, dietary supplementation of 40, 60, and 80 mg/kg Fe did not influence ($P > 0.05$) the feed intake, egg mass, feed conversion ratio, and egg production but significantly influenced ($P < 0.05$) the egg weight of the breeder geese during the experiment.

Table 2. Effects of iron (Fe) and *Bacillus subtilis* supplementation on production performance of breeder geese.[1]

Treatment						
Fe (mg/kg)	*B. subtilis* (CFU/kg)	Feed intake (g/hen per D)	Egg mass (g/hen per D)	Feed conversion ratio (g of feed/g of egg)	Egg production (%)	Egg weight (g)
40	2.5×10^9	220.51	26.68	9.04	28.13	128.98
60	2.5×10^9	220.30	25.38	7.77	26.13	131.48
80	2.5×10^9	220.77	37.27	6.51	24.93	131.15
40	5.0×10^9	220.72	34.70	6.44	27.11	128.65
60	5.0×10^9	220.11	26.94	6.42	31.07	132.01
80	5.0×10^9	220.34	24.59	9.18	27.91	133.23
SEM		0.32	4.88	1.23	0.01	2.82
Main effect						
Fe (mg/kg)	40	220.62	30.69	7.74	27.62	128.82[b]
	60	220.21	26.16	7.10	28.60	131.75[a]
	80	220.55	30.93	7.85	26.42	132.19[a]
B. subtilis (CFU/kg)	2.5×10^9	220.53	29.78	7.78	26.40[b]	131.29
	5.0×10^9	220.39	28.74	7.35	28.70[a]	130.54
P-value						
Fe		0.845	0.557	0.806	0.194	0.036
B. subtilis		0.830	0.798	0.674	0.025	0.482
Fe × *B. subtilis*		0.914	0.124	0.109	0.052	0.645

[a,b]Means within the same column with different superscripts differ significantly ($P < 0.05$).
[1]Data represent the means of 4 replicates cages per treatment.

Dietary supplementation of 60 and 80 mg/kg Fe significantly increased the egg weight compared with that of 40 mg/kg Fe ($P < 0.05$). Dietary *B. subtilis* supplementation did not influence ($P > 0.05$) the feed intake, egg mass, feed conversion ratio, and egg weight but significantly influenced ($P < 0.05$) the egg production. Dietary supplementation of 5.0×10^9 CFU/kg *B. subtilis* significantly increased the egg production by 2.3% compared with 2.5×10^9 CFU/kg *B. subtilis*-supplemented groups ($P < 0.05$). There was no interaction between dietary Fe and *B. subtilis* supplementation on all of the measured production performance indexes ($P > 0.05$).

Hatching Performance

A significant interaction on the percentage of hatched eggs was observed between dietary Fe and *B. subtilis* supplementation ($P < 0.05$; Table 3). The geese-fed diets supplemented with 60 mg/kg Fe and 5×10^9 CFU/kg *B. subtilis* showed the highest percentage of hatched eggs (97.50%) and the geese-fed diets supplemented with 40 mg/kg Fe and 2.5×10^9 CFU/kg *B. subtilis* showed the lowest percentage of hatched eggs (85.00%). Compared with the groups with Fe supplementation at 40 mg/kg, providing 60 and 80 mg/kg Fe to breeder geese significantly increased fertility ($P < 0.05$). No significant influence was observed on the hatchability by the main effects and their interaction ($P > 0.05$).

Egg Quality

Dietary supplementation of 5.0×10^9 CFU/kg *B. subtilis* significantly increased the eggshell thickness by

Table 3. Effects of iron (Fe) and *Bacillus subtilis* supplementation on hatching performance of breeder geese[1].

Treatment				
Fe (mg/kg)	*B. subtilis* (CFU/kg)	Percentage of hatched eggs (%)	Fertility (%)	Hatchability (%)
40	2.5×10^9	85.00[d]	86.76	88.21
60	2.5×10^9	90.00[c]	91.67	92.56
80	2.5×10^9	92.50[b,c]	91.89	94.28
40	5.0×10^9	95.00[a,b]	89.60	92.65
60	5.0×10^9	97.50[a]	93.62	95.91
80	5.0×10^9	92.50[b,c]	91.96	92.65
SEM		0.05	0.04	0.01
Main effect				
Fe (mg/kg)	40	90.00	88.18[b]	90.43
	60	93.75	92.64[a]	94.23
	80	92.50	91.92[a]	93.46
B. subtilis (CFU/kg)	2.5×10^9	89.17	90.11	91.68
	5.0×10^9	95.00	91.73	93.73
P-value				
Fe		0.032	0.022	0.135
B. subtilis		<0.001	0.217	0.202
Fe × *B. subtilis*		0.004	0.668	0.260

[a–d]Means within the same column with different superscripts differ significantly ($P < 0.05$).
[1]Data of percentage of hatched eggs represent the means of 4 replicates cages per treatment. Data of fertility and hatchability represent the means of 4 replicates, with 5 eggs per replicate.

8.3% compared with 2.5×10^9 CFU/kg *B. subtilis*-supplemented groups ($P < 0.05$; Table 4). The main effect of dietary Fe supplementation and their interaction did not affect eggshell thickness ($P > 0.05$). Different levels of Fe and *B. subtilis* and their interactions did not result in changes on the egg shape index, eggshell strength, albumen height, yolk color, Haugh unit, and yolk ratio ($P > 0.05$).

Apparent Nutrient Digestibility

As shown in Table 5, *B. subtilis* addition at 5.0×10^9 CFU/kg significantly increased the apparent digestibility of P compared with *B. subtilis* addition at 2.5×10^9 CFU/kg ($P < 0.05$). There was a significant interaction between dietary Fe and *B. subtilis* supplementation on the apparent digestibility of Ca ($P < 0.05$). In the bird fed 5.0×10^9 CFU/kg *B. subtilis*, 40 and 60 mg/kg Fe supplementation significantly increased the apparent digestibility of Ca compared with 80 mg/kg Fe supplementation. However, in the bird fed 2.5×10^9 CFU/kg *B. subtilis*, Fe supplementation did not affect the apparent digestibility of Ca. No significant difference was found on the apparent digestibility of crude protein, ether extract, and crude fiber by the main effects and their interaction ($P > 0.05$).

Serum Hormone and Urea Nitrogen Concentrations

The serum FSH level was significantly higher in geese fed diets supplemented with 5×10^9 CFU/kg *B. subtilis* than that in geese fed diets supplemented with 2.5×10^9 CFU/kg *B. subtilis* ($P < 0.05$; Table 6). The main effect of dietary Fe supplementation and their interaction did not affect serum FSH level ($P > 0.05$). No significant difference was seen on the serum E2 and prolactin levels by the main effects and their interaction ($P > 0.05$). There was a significant interaction between dietary Fe and *B. subtilis* supplementation on the serum urea nitrogen level ($P < 0.05$). Group supplemented with 60 mg/kg Fe and 5×10^9 CFU/kg *B. subtilis* and group supplemented with 80 mg/kg Fe and 5×10^9 CFU/kg *B. subtilis* significantly decreased serum urea nitrogen level compared with other 4 groups.

Serum Antioxidant Parameters

As shown in Table 7, the T-AOC in the 60 and 80 mg/kg Fe-supplemented groups were significantly higher than that in the 40 mg/kg Fe-supplemented groups. Dietary *B. subtilis* supplementation at 5.0×10^9 CFU/kg significantly increased ($P < 0.05$) serum T-AOC and significantly decreased ($P < 0.05$) MDA level compared with *B. subtilis* supplementation at 2.5×10^9 CFU/kg.

Hematological Parameters

Dietary Fe supplementation significantly elevated the RBC, HCT, HGB, and MCV, regardless of *B. subtilis* supplementation ($P < 0.05$; Table 8). Red blood cells, HCT, and MCV in the 60 mg/kg Fe-supplemented groups were significantly higher than those in the 40 and 80 mg/kg Fe-supplemented groups ($P < 0.05$). Dietary supplementation of 5.0×10^9 CFU/kg *B. subtilis* significantly increased the HCT, HGB, and MCV compared with 2.5×10^9 CFU/kg *B. subtilis*-supplemented groups ($P < 0.05$). There was a significant interaction between dietary Fe and *B. subtilis* supplementation on the MCH ($P < 0.05$). Geese fed diets supplemented with 60 mg/kg Fe and 5×10^9 CFU/kg *B. subtilis* showed the highest MCH among the 6 groups ($P < 0.05$).

DISCUSSION

The present study revealed that dietary *B. subtilis* supplementation at 5.0×10^9 CFU/kg significantly

Table 4. Effects of iron (Fe) and *Bacillus subtilis* supplementation on egg quality of breeder geese.[1]

Treatment								
Fe (mg/kg)	*B. subtilis* (CFU/kg)	Egg shape index	Eggshell strength (kg/cm^2)	Eggshell thickness (mm)	Albumen height (mm)	Yolk color	Haugh unit	Yolk ratio (%)
40	2.5×10^9	1.46	5.026	0.484	15.68	2.45	118.85	42.43
60	2.5×10^9	1.51	5.069	0.494	15.90	2.60	120.15	39.14
80	2.5×10^9	1.46	4.981	0.465	14.90	2.40	119.35	36.45
40	5.0×10^9	1.50	5.032	0.519	15.73	2.50	121.85	39.61
60	5.0×10^9	1.50	5.098	0.540	17.20	2.70	122.55	41.74
80	5.0×10^9	1.51	5.065	0.504	15.55	2.65	118.93	37.77
SEM		0.01	0.15	0.02	0.74	0.11	2.98	0.03
Main effect								
Fe (mg/kg)	40	1.48	5.029	0.502	15.70	2.48	120.35	41.02
	60	1.50	5.083	0.517	16.55	2.65	121.35	40.44
	80	1.48	5.023	0.485	15.23	2.53	119.14	37.11
B. subtilis (CFU/kg)	2.5×10^9	1.48	5.025	0.481[b]	15.49	2.48	119.45	39.34
	5.0×10^9	1.51	5.065	0.521[a]	16.16	2.62	121.11	39.71
P-value								
Fe		0.736	0.239	0.274	0.122	0.384	0.164	0.275
B. subtilis		0.261	0.233	0.020	0.202	0.215	0.083	0.861
Fe × *B. subtilis*		0.527	0.587	0.957	0.607	0.719	0.282	0.547

[1]Data represent the means of 4 replicates, with 3 eggs per replicate.

Table 5. Effects of iron (Fe) and *Bacillus subtilis* supplementation on nutrient digestibility of breeder geese.[1]

Treatment						
Fe (mg/kg)	*B. subtilis* (CFU/kg)	CP (%)	EE (%)	CF (%)	Ca (%)	P (%)
40	2.5×10^9	67.62	64.80	20.12	43.57[b]	40.26
60	2.5×10^9	68.00	65.31	15.71	43.81[b]	40.97
80	2.5×10^9	69.22	64.72	21.15	44.95[a,b]	39.54
40	5.0×10^9	67.70	64.34	23.92	46.23[a]	41.81
60	5.0×10^9	70.84	67.13	26.65	45.96[a]	42.63
80	5.0×10^9	68.51	64.50	28.15	44.27[b]	42.45
SEM		0.02	0.01	0.05	0.44	0.43
Main effect						
Fe (mg/kg)	40	67.67	64.57	22.02	44.90	41.03
	60	69.42	66.22	21.19	44.88	41.79
	80	68.88	64.61	19.65	44.61	40.99
B. subtilis (CFU/kg)	2.5×10^9	68.28	64.95	19.00	44.11	40.26[b]
	5.0×10^9	69.02	65.32	22.91	45.49	42.29[a]
P-value						
Fe		0.787	0.376	0.910	0.780	0.146
B. subtilis		0.731	0.728	0.398	0.002	<0.001
Fe × *B. subtilis*		0.773	0.640	0.467	0.004	0.246

[a,b]Means within the same column with different superscripts differ significantly ($P < 0.05$).
Abbreviations: Ca, calcium; CF, crude fiber; CP, crude protein; EE, ether extract; P, total phosphorus.
[1]Data represent the means of 4 replicates, with 1 female goose per replicate.

increased egg production of breeder geese by 2.3% compared with *B. subtilis* supplementation at 2.5×10^9 CFU/kg during the entire experimental period. It has been reported that dietary *B. subtilis* supplementation increased egg production of laying hens (Chen et al., 2019; Yang et al., 2019). *B. subtilis* supplementation did not affect feed intake and feed conversion ratio of breeder geese, which similar to the results of Chen et al. (2019) and Sobczak and Kozłowski (2015) in laying hens. In the present study, dietary supplementation of 60 and 80 mg/kg Fe increased the egg weight compared with 40 mg/kg Fe supplementation. Similarly, Paik et al. (2009) reported that the egg weight was promoted by dietary iron–soy proteinate supplementation compared with the un-supplemented control laying hens.

In our study, a significant interaction between dietary Fe and *B. subtilis* supplementation was observed on percentage of hatched eggs. Geese fed 60 mg/kg Fe and 5.0×10^9 CFU/kg *B. subtilis* had the greatest percentage of hatched eggs, which indicated that dietary Fe and *B. subtilis* supplementation synergistically increased the percentage of hatched eggs. Our results showed that breeder geese fed 60 mg/kg Fe diets had the highest fertility, 4.46% greater than those fed 40 mg/kg Fe diets. Iron is a critical trace nutrient for animal reproduction. Excess or deficiency of iron may reduce libido, impair spermatogenesis, and cause oxidative damage to the spermatozoa and testicular tissue, ultimately leading to fertility damage (Toebosch et al., 1987; Wise et al., 2003).

Table 6. Effects of iron (Fe) and *Bacillus subtilis* supplementation on serum hormone and urea nitrogen levels of breeder geese.[1]

Treatment					
Fe (mg/kg)	*B. subtilis* (CFU/kg)	FSH (mIU/mL)	E2 (pg/mL)	PRL (mIU/L)	Urea nitrogen (mmol/L)
40	2.5×10^9	0.79	94.67	131.88	3.77[a]
60	2.5×10^9	1.65	158.48	268.90	3.44[a]
80	2.5×10^9	1.09	116.41	138.57	3.44[a]
40	5.0×10^9	1.32	221.46	373.23	3.73[a]
60	5.0×10^9	3.40	174.87	401.70	1.87[b]
80	5.0×10^9	1.89	113.42	129.00	2.42[b]
SEM		0.33	28.77	31.32	0.23
Main effect					
Fe (mg/kg)	40	1.06	158.07	252.56	3.75
	60	2.53	166.68	335.30	2.65
	80	1.49	114.91	131.79	2.93
B. subtilis (CFU/kg)	2.5×10^9	1.18[b]	123.19	179.78	3.55
	5.0×10^9	2.21[a]	169.91	301.31	2.67
P-value					
Fe		0.054	0.466	0.195	0.001
B. subtilis		0.043	0.209	0.181	<0.001
Fe × *B. subtilis*		0.551	0.305	0.513	0.022

[a,b]Means within the same column with different superscripts differ significantly ($P < 0.05$).
Abbreviations: E2, estradiol; FSH, follicle stimulating hormone; PRL, prolactin.
[1]Data represent the means of 4 replicates, with 2 female geese per replicate.

Table 7. Effects of iron (Fe) and *Bacillus subtilis* supplementation on serum antioxidant capacity of breeder geese.[1]

Treatment					
Fe (mg/kg)	*B. subtilis* (CFU/kg)	T-AOC (U/mL)	CAT (U/mL)	T-SOD (U/mL)	MDA (nmol/mL)
40	2.5×10^9	8.30	1.23	297.72	1.79
60	2.5×10^9	11.91	1.24	327.37	1.72
80	2.5×10^9	13.24	1.5	345.52	1.54
40	5.0×10^9	14.12	1.18	378.2	1.71
60	5.0×10^9	19.11	2.01	364.89	0.69
80	5.0×10^9	15.90	1.25	329.41	1.04
SEM		1.36	0.21	28.62	0.23
Main effect					
Fe (mg/kg)	40	11.21[b]	1.21	337.96	1.75
	60	15.51[a]	1.62	346.13	1.21
	80	14.57[a]	1.38	337.47	1.29
B. subtilis (CFU/kg)	2.5×10^9	11.15[b]	1.32	323.54	1.68[a]
	5.0×10^9	16.38[a]	1.48	357.5	1.14[b]
P-value					
Fe		0.022	0.161	0.949	0.053
B. subtilis		<0.001	0.381	0.181	0.008
Fe × *B. subtilis*		0.308	0.054	0.294	0.133

[a,b]Means within the same column with different superscripts differ significantly ($P < 0.05$).

Abbreviations: CAT, catalase; MDA, malondialdehyde; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase.

[1]Data represent the means of 4 replicates, with 2 female geese per replicate.

Our study showed that *B. subtilis* supplementation contributed to an improvement in eggshell thickness in breeder geese. Dried *B. subtilis* cultures have been reported to increase eggshell thickness of laying hens (Xu et al., 2006). Abdelqader et al. (2013) found that *B. subtilis* supplementation increased eggshell quality (including eggshell weight, eggshell thickness, eggshell density, and unmarketable eggs) of laying hens. However, Neijat et al. (2019) reported that dietary *B. subtilis* supplementation showed no evident effect on eggshell quality in laying hens. The discrepancy may be because of the differences in bacterial strain, supplemental dosage, sampling time, and experimental animals. In the current study, *B. subtilis* supplementation improved apparent digestibility of Ca and P in breeder geese. It is reported that the beneficial effects of probiotics on egg shell quality are in connection with the improvement of mineral availability, particularly, Ca and P (Abdelqader et al., 2013; Świątkiewicz et al., 2014). This may be attributable to a favorable environment in the intestinal tract after probiotics administration. Probiotic bacteria increase the fermentation rate and promote the production of short-chain fatty acids, which reduces the luminal pH (Scholz-Ahrens et al., 2007). Low luminal pH helps to solubilize more Ca and P (van den Heuvel et al., 1999). Thus, dietary *B. subtilis* can improve the eggshell quality through increasing Ca and P availability.

The egg-laying performance is controlled by the rate of growth and differentiation of ovarian follicles that

Table 8. Effects of iron (Fe) and *Bacillus subtilis* supplementation on hematological parameters of breeder geese.[1]

Treatment						
Fe (mg/kg)	*B. subtilis* (CFU/kg)	RBC (10^{12}/L)	HCT (%)	HGB (g/L)	MCV (fL)	MCH (pg)
40	2.5×10^9	3.29	36.25	154.08	118.58	68.43[c]
60	2.5×10^9	3.64	37.75	158.90	122.48	69.93[b]
80	2.5×10^9	3.50	35.90	158.93	119.88	71.03[b]
40	5.0×10^9	3.50	37.15	161.98	119.88	67.40[c]
60	5.0×10^9	3.74	40.75	166.93	124.83	72.90[a]
80	5.0×10^9	3.41	37.55	166.08	119.98	70.15[b]
SEM		0.07	0.51	1.34	0.65	0.41
Main effect						
Fe (mg/kg)	40	3.39[b]	36.70[b]	158.03[b]	119.23[b]	67.91
	60	3.69[a]	39.25[a]	162.91[a]	123.65[a]	71.41
	80	3.46[b]	36.73[b]	162.50[a]	119.93[b]	70.59
B. subtilis (CFU/kg)	2.5×10^9	3.48	36.63[b]	157.3[b]	120.31[b]	69.79
	5.0×10^9	3.55	38.48[a]	164.99[a]	121.56[a]	70.15
P-value						
Fe		0.001	<0.001	0.005	<0.001	<0.001
B. subtilis		0.233	<0.001	<0.001	0.025	0.309
Fe × *B. subtilis*		0.122	0.138	0.946	0.228	<0.001

[a–c]Means within the same column with different superscripts differ significantly ($P < 0.05$).

Abbreviations: HCT, hematocrit; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; RBC, red blood cell.

[1]Data represent the means of 4 replicates, with 2 female geese per replicate.

are, in turn, under the regulation of reproductive hormones such as FSH, E2, progesterone, and growth factors (Onagbesan et al., 2006). Follicle stimulating hormone is the essential hormone to follicular growth, development, dominance, maturation, and ovulation. Estradiol has a feedback effect on the hypothalamus and pituitary to promote follicular development (Tarumi et al., 2014). Prolactin plays a stimulatory role in ovarian follicular development and egg laying in chicken hens (Li et al., 2011a). Accordingly, the serum hormone level has been considered as a sensitive indicator of laying performance (Mohammadi and Ansari-Pirsaraei, 2014). In our study, the increase of serum FSH level by dietary *B. subtilis* supplementation may be contributed to the elevated egg production. Blood urea nitrogen and uric acid are the products of protein metabolism of poultry and are eliminated from the body by the kidneys. Blood urea nitrogen reflects the balance between proteins and amino acids of an animal, in that a well-balanced amino acid concentration leads to a decreased blood urea nitrogen concentration (Li et al., 2011b). In the present study, supplementation with 60 and 80 mg/kg Fe in the birds fed 5×10^9 CFU/kg *B. subtilis* significantly decreased serum urea nitrogen level compared with other 4 groups, which indicated that appropriate Fe and *B. subtilis* supplementation could synergistically improve the efficiency of protein utilization.

The T-AOC is a comprehensive index for measuring the antioxidant capacity of both enzymatic and nonenzymatic defense systems (Birben et al., 2012). Our results showed that Fe added to diets contributed to an improvement of T-AOC in serum. Iron is an essential micronutrient, but the continuous presence of an excessive intake of Fe would produce reactive oxygen species (Nicholls and Budd, 2000; Zödl et al., 2003), which could produce pathological changes through lipid peroxidation and DNA damage. The results of our study indicated that dietary Fe addition contributed to the elevation of antioxidant capability of breeder geese, which was agreed with other studies performed in laying hens (Xie et al., 2019) and stinging catfish (Zafar and Khan, 2020). The important components of the antioxidative enzymes, including total superoxide dismutase and catalase, play a crucial role in eliminating superoxide anions and hydrogen peroxide and offering protection against damage of cells and tissues by oxidative stress (Fukai and Ushio-Fukai, 2011; Birben et al., 2012). Malondialdehyde is a good indicator of oxidative tissue damage, which is normally accompanied by the reduction of antioxidant capacity. Numerous studies reported that dietary probiotics were beneficial in oxidation resistance, scavenging ROS, and promoting antioxidant capability in chicken (Abudabos et al., 2016; Bai et al., 2016). In the present study, increased T-AOC and decreased MDA content were evident with the higher addition of *B. subtilis* in the diets. These results were in agreement with previous findings in showing that addition of *B. subtilis* had a positive response on antioxidant activity in serum of broilers in reports by Rajput et al. (2013) and Bai et al. (2017).

Hematological analyses may provide a reflection of the health status of animals. Iron participates in oxygen transport as an important part of hemoglobin and myoglobin molecules (Bess et al., 2012). Blood RBC, HGB, and HCT are traditional parameters used to evaluate Fe metabolism and nutritional status of host because dietary Fe directly impacted their values. In the present study, geese fed diets containing 60 mg/kg Fe exhibited significant increases in the values of RBC, HCT, HGB, and MCV compared with those fed diets containing 40 mg/kg Fe. It was reported that dietary Fe supplementation elevated HCT and HGB values of broiler breeder hens and their progeny (Taschetto et al., 2017). In our study, dietary supplementation with *B. subtilis* at 5.0×10^9 CFU/kg improved the values of HCT, HGB, and MCV. Similar findings have been reported that the values of HCT and HGB were higher in fish fed diets supplemented with *B. subtilis* (Hassaan et al., 2018). Zhu et al. (2019) also found that the values of HCT were higher in fish fed *B. subtilis*-supplemented diets than that in fish fed control diet. The results of our study indicated that the application of Fe and *B. subtilis* improved the hematopoietic system of breeder geese.

In conclusion, the combined use of Fe and *B. subtilis* in the diet contributed to enhancing the reproductive performance, eggshell quality, nutrient digestibility, antioxidant status, and hematopoietic function of breeder geese. Based on the results of our study, dietary addition of 60 mg/kg Fe and 5.0×10^9 CFU/kg *B. subtilis* is regarded as an optimal level for improving reproductive performance in breeder geese.

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