Effects of zinc and *Bacillus subtilis* on the reproductive performance, egg quality, nutrient digestion, intestinal morphology, and serum antioxidant capacity of geese breeders

Wenlei Fan ,[*,1] Jing Shi,[*,1] Baowei Wang,[†,2] Mingai Zhang,[†] Min Kong,[‡] and Wenli Li[*]

[*]*College of Animal Science and Technology, Qingdao Agricultural University, Qingdao 266109, People's Republic of China;* [†]*College of Food Science and Engineering, Qingdao Agricultural University, Qingdao 266109, People's Republic of China; and* [‡]*Institute of high quality waterfowl, Qingdao Agricultural University, Qingdao 266109, People's Republic of China*

ABSTRACT The effects of zinc (**Zn**) and *Bacillus subtilis* (***B. subtilis***) on reproductive performance, egg quality, nutrient digestion, intestine morphology, and antioxidant capacity were explored in geese breeders. Geese breeders (n = 120, 46-wk of age) were randomly assigned into 6 groups with 4 replicates of 5 birds each (1 male and 4 female). Breeders were fed diets with 2 levels of *B. subtilis* (2.5×10^9 and 5×10^9 CFU/kg) crossed with three levels of Zn (25, 45, and 65 mg/kg) for duration of 10-wk. The results showed that the egg laying rate ($P < 0.05$), fertility rate ($P < 0.01$), hatchability rate ($P < 0.05$), yolk color ($P < 0.05$), and the retentions of crude protein ($P < 0.05$), ether extract ($P < 0.05$) and phosphorus of geese breeders were improved by dietary supplementation of 5×10^9 CFU/kg *B. subtilis* and 25 mg or 45 mg/kg Zn. The serum T-SOD ($P < 0.05$) was increased by 45 mg/kg Zn supplementation. The serum T-AOC ($P < 0.05$) and retention of Zn ($P < 0.05$) were increased by 5×10^9 CFU/kg *B. subtilis* supplementation. The birds fed with 5×10^9 CFU/kg *B. subtilis* and 25 mg or 45 mg/kg Zn showed improved villus length ($P < 0.01$) and villus length/ crypt depth ($P < 0.01$) in both the jejunum and ileum. In conclusion, the combination of *B. subtilis* and Zn may have synergistic effects on these parameters, and dietary inclusion of 5×10^9 CFU/kg *B. subtilis* and 45 mg/kg Zn is recommended for improving the reproductive performance of geese breeders.

Key words: geese breeder, zinc, *Bacillus subtilis*, reproductive performance, intestinal morphology

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INTRODUCTION

Zinc (**Zn**) is one of the essential trace mineral of poultry nutrition, and act as component of more than 300 enzymes that are involved in the metabolism of energy and protein (Jurowski et al., 2014; Huang et al., 2019). Zn is required for growth, reproduction, bone development, and intestinal health (Jankowski et al., 2019; De Grande et al., 2020). The concentration and bioavailability of Zn in many feed-stuffs for poultries are low, dietary supplementation with Zn is usually recommended. The beneficial effects of dietary Zn supplementation on egg production, egg quality, hatchability, embryonic development, and offspring's performance have been reported in layer and breeding birds (Liao et al., 2018; Mayer et al., 2019).

In commercial poultry diets, Zn is usually supplemented in forms of Zn oxide or Zn sulphate to meet the Zn requirements of broiler chicken, as suggested by various feeding standards (Hudson et al., 2005; Abd El-Hack et al., 2017). However, the absorption and bioavailability of inorganic Zn are quite low, so excessive level of inorganic Zn was widely used in poultry diets (Abd El-Hack et al., 2017). The excreted Zn could pollute soils and water, after Zn-rich manure from poultry is applied to the fields. Pollution with metals elements, like Cu and Zn, has a potential hazardous effect not only on crop plants but also on human health (Kuang et al., 2015; VanValin et al., 2018). Therefore, there is a growing interest in finding ways to improve the utilization of Zn in poultry feeds.

Bacillus subtilis is a probiotic that has been widely used to improve the performance of broiler chicks and laying hens (Li et al., 2016; Gao et al., 2017). It has been reported that dietary *B. subtilis* supplementation

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[1]Both authors contributed equally to this study.

[2]Corresponding author: wangbw@qau.edu.cn

increased the egg production, feed conversion ratio, and egg quality in hens, which was proposed to be associated with lowering gastrointestinal pH and an inhibitory effect on bacterial enzyme activities (Neijat et al., 2019). The mechanisms of the improvement of hen performance by the probiotics may also include improving intestinal health and utilization of nutrients, by maintaining the gut integrity and enhancing the antioxidant capacity (Elshaghabee et al., 2017; Rose et al., 2018). A previous study in our laboratory suggested that dietary *B. subtilis* supplementation increased the apparent retention of Zn in meat-type geese (Ke et al., 2018). Thus, we hypothesized that the level of inorganic Zn supplementation in poultry feeds can be reduced by the combined use of *B. subtilis*.

The Zn recommendations published in diets for commercial laying hens is 45 mg Zn/kg of feed by the NRC (1994), and varies from 65 to 110 mg Zn/kg of feed in other sources (Mayer et al., 2019; Zhang et al., 2020). Despite the physiological importance of Zn for poultry production, Zn requirements of geese breeders were not covered in NRC (1994) and remains to be estimated. A recent study in our laboratory has demonstrated that the dietary Zn requirement for goose breeder was 65 to 70 mg Zn/kg of feed (Shi et al., 2019). The objective of the present study was to test if the dietary Zn level could be reduced by *B. subtilis* supplementation in laying goose breeder, as being assessed with the reproductive performance, egg quality, nutrient utilization, intestinal morphology, and antioxidant capacity.

MATERIALS AND METHODS

The use of the birds and all experimental procedures with geese were approved by the animal ethics committee of Qingdao Agricultural University, according to the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China).

Birds, Experimental Design, Diets, and Rearing Conditions

A total of 120 forty five-wk-old Wulong goose breeders (*Anser cygnoides*, a local goose breed) with comparable body weight (**BW**), from the breeding center of Qingdao Agricultural University, were randomly allocated to 6 groups, with 4 replicates (pens) of 5 birds (1 male and 4 female) in each pen. A 2 × 3 factorial experimental design was applied to this experiment. Two levels of *B.* subtilis levels (2.5×10^9 and 5×10^9 CFU/kg) were crossed with 3 levels of Zn (25, 45, and 65 mg/kg), and supplemented into the basal diet.

The basal diets were formulated mainly with corn and soybean meal, according to the recommendation of NRC (1994) for geese breeders. The composition and nutrient levels of the basal diet are listed in Table 1. Supplementation was done using laboratory grade Zn sulfate heptahydrate ($ZnSO_4 \bullet 7H_2O$) (Sigma Aldrich, St. Louis, MO). *Bacillus subtilis* were provided by a commercial supplier (Puxing Biological Technology Co., Ltd., Qingdao, China). From 45 to 46 wk of age, birds received a Zn-deficient diet (the basal diet, 20.64 Zn mg/kg as analyzed), to deplete the body Zn storage. Then, birds were fed with 6 experimental diets for 10 wk.

Table 1. Composition and nutrient levels of basal diet for geese breeders (as fed basis).

Ingredients	%	Nutrient composition	%
Corn	59.45	Crude protein	16.30
Soybean meal	19.45	Metabolizable energy (MJ/kg)	11.40
Fish meal	3.00	Calcium	2.89
Wheat bran	0.50	Available phosphorus	0.47
Calcium phosphate	1.25	Lysine	0.85
Limestone	5.58	Methionine	0.45
Rice hull	7.70	Cystine	0.25
Soybean oil	1.90	Methionine + cystine	0.70
NaCl	0.40	Arginine	0.95
Trace mineral premix[1]	0.50	Crude fiber[2]	4.03
Vitamin premix[1]	0.11	Zn (mg/kg)[2]	20.64
DL-Methionine (99%)	0.16		
Total	100		

[1]Supplied per kilogram of total diet: Cu ($CuSO_4 \bullet 5H_2O$), 4 mg; Fe ($FeSO_4 \bullet {}_7H_2O$), 80 mg; Mn ($MnSO_4 \bullet H_2O$), 30 mg; Se ($NaSeO_3$), 0.5 mg; I (KI), 0.3 mg; choline chloride, 750 mg; vitamin A (retinyl acetate), 9,000 IU; vitamin D_3 (Cholcalciferol), 2,000 IU; vitamin E (DL-α-tocopheryl acetate), 40 IU; vitamin K_3 (menadione sodium bisulphate), 0.8 mg; thiamin (thiamin mononitrate), 2 mg; riboflavin, 4 mg; pyridoxine hydrochloride, 4 mg; cobalamin, 0.012 mg; calcium-D-pantothenate, 11 mg; nicotinic acid, 30 mg; folic acid, 0.5 mg; and biotin, 0.2 mg.

[2]These values determined by analysis based on triplicate determinations.

All geese were reared in plastic wire-floor pens (12 m × 1.24 m) and had ad libitum access to feed and water during the experimental period. Water was provided by the same half-open, plastic, cylindrical water tank, and powdered feed was provided in feed troughs on one side of the pen. The geese were maintained under natural daylight condition.

Reproductive Performance

The eggs were collected daily from each pen and the number was recorded. Eggs were stored at 16°C under relative humidity of 75%. Egg laying rate (%, the number of eggs/the number of breeders) was calculated daily for each per replicate. During the last 2 wk of experiment, 5 eggs from each replicate were randomly selected and incubated in a commercial incubator (Yiai 12,096, Qingdao, China). The eggs were candled on d 6 and 18 to eliminate infertile eggs and dead embryos. At the end of incubation, the eggs that failed to hatch out and the hatched goslings were counted. The hatchability of fertile eggs was calculated as the percentage of the number of hatched goslings to the number of fertile eggs. Egg fertility and hatchability were also calculated.

Egg Quality

Eggs were collected during the last 2 wk of the experiment. Eight eggs per replicate were randomly selected for egg quality tests, including eggshell strength, eggshell thickness, yolk color, and Haugh unit. Fresh eggs were

stored at 16°C under relative humidity of 75% lesser than 24 h before the tests. The length and width of the eggs were measured using the digital caliper, and the egg shape index was calculated as width × 100/length. Eggshell thickness was measured at the two pole ends and at the middle section of the egg shell with a digital micrometer (NABEL Co. Ltd., Kyoto, Japan). The other tests were performed using an egg Analyzer (DET-6000, NABEL Co. Ltd.) following the manufacturer's instructions.

Nutrient Utilization

During the last week of the experiment, one female goose from each replicate was randomly selected and all geese selected were kept in individual metabolic cages. Birds were fasted for 24-h, then each bird was fed with 120 g/day of the experiment diets for 3 d. The excreta samples from each cage were collected over 3 consecutive days, and fecal samples were pooled for each cage. Feed samples and dried excreta samples at 65°C were ground and analyzed for contents of crude fiber, crude protein, crude fat (ether extract), calcium (**Ca**), phosphorus (**P**), and Zn according to the AOAC (2005) methods. The Ca, P, and Zn content in feed and excreta samples were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES-Optima 8 × 00; PerkinElmer Inc., Alpharetta, GA).

Serum and Intestinal Sample Collection

At the end of the experiment, one goose was randomly selected from each pen and fasted for 12 h. Then, blood sample was drawn from the wing vein into a coagulant tube and centrifuged at 2,500 × *g* for 10 min at 4°C to obtain serum. Serum samples were stored at −20°C until analysis. The geese selected were then euthanized by cervical dislocation. The small intestine (jejunum and ileum) samples were collected, rinsed with saline and fixed in 4% buffered formaldehyde for histological study.

Intestinal Histological Study

The intestine samples were embedded in paraffin blocks, after dried up using a graded series of xylene and ethanol. Then, the jejunum and ileum sections (5 μm) were stained with hematoxylin and eosin (**H&E**). Ten slides for each sample (the middle site of the sample) were prepared, and the images were acquired using an OLYMPUS microscope (OLYMPUS, Japan). The villus length (**VL**) and crypt depth (**CD**) were measured from 6 different views per slide according to the method by Shah et al. (2019). The villus length to crypt depth ratio (**VL/CD**) was calculated.

Antioxidant Capacity Analysis

The total superoxide dismutase (**T-SOD**) and total antioxidant capacity (**T-AOC**) in serum samples were assayed using colorimetric methods with commercial kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing, Jiangsu, China), according to manufacture's protocols. The values were expressed as units per milliliter for serum.

Statistical Analysis

Data were analyzed using the two-way analysis of variance (ANOVA) protocol in SPSS 18.0 (Statistical Package for Social Science; SPSS Inc., Chicago, IL). Zinc and *B. subtilis* were factors, and their interaction was included in the model. When the interaction was not significant ($P > 0.05$), data was re-analyzed in the model excluding the interaction and are presented for Zn and *B. subtilis*, respectively. Replicates were considered the experimental units. Pooled SEMs were calculated by averaging the SEMs calculated with least significant difference (**LSD**) to identify differences. Means were compared by using LSD multiple range tests. Statistical significance was declared for $P < 0.05$.

RESULTS

Reproductive Performance

The effects of dietary supplementation of Zn and *B. subtilis* on reproductive performance of laying goose breeders are shown in Table 2. Significant interactions between Zn and *B. subtilis* were observed on the egg production, fertility rate, and hatchability rate ($P < 0.05$). Goose breeders fed the diets supplemented with 5×10^9 CFU/kg *B. subtilis* and 45 mg/kg Zn showed the highest egg laying rate, fertility rate, and hatchability rate. The Zn level and the interaction between Zn and B. subtilis did not affect the healthy gosling rate ($P > 0.05$). Compared with *B. subtilis* supplementation at 2.5×10^9 CFU/kg, supplementation of *B. subtilis* at 5.0×10^9 CFU/kg significantly increased the healthy gosling rate (97.6% vs. 84.0%, $P < 0.05$).

Egg Quality

As shown in Table 3, no significant effects of *B. subtilis* and Zn supplementations and their interactions were noted on the, eggshell strength, eggshell thickness, albumen height, and Haugh unit ($P > 0.05$). A significant interaction between Zn and *B. subtilis* was observed on yolk color ($P < 0.05$). Eggs from goose breeders fed the diet supplemented with 5.0×10^9 CFU/kg *B. subtilis* and 45 mg/kg Zn showed the greatest value of yolk color.

Digestion and Utilization of Nutrients

Significant interactions between Zn and *B. subtilis* were observed on the retentions of ether extract, crude protein, and phosphorus ($P < 0.05$), as shown in Table 4. Goose breeders fed the diets supplemented with 5×10^9

Table 2. Effects of Zn and *Bacillus subtilis* supplementations on the reproductive performance of goose breeder[1].

Treatment					
Bacillus subtilis (CFU/kg)	Zn (mg/kg)	Egg production (%)	Fertility (%)	Hatchability (%)	Healthy gosling (%)
5×10^9	25	35.45[ab]	66.25[a]	89.01[b]	98.08
	45	39.91[a]	70.00[a]	98.44[a]	100.00
	65	33.39[b]	55.00[b]	86.70[b]	94.72
2.5×10^9	25	26.68[c]	38.75[c]	68.30[c]	71.67
	45	31.02[bc]	50.00[b]	82.50[bc]	91.32
	65	33.37[b]	52.50[b]	90.45[b]	89.17
	Pooled SEM	1.55	1.91	4.32	3.35
Main effect					
	25	31.07	52.50	78.66	84.87
	45	35.46	60.00	90.47	95.66
	65	33.38	53.75	88.58	91.94
	Pooled SEM	1.10	1.35	3.06	2.37
5.0×10^9		36.25	63.75	91.38	97.60[a]
2.5×10^9		30.35	47.08	80.42	84.05[b]
	Pooled SEM	0.89	1.10	2.49	1.93
P-value					
Zn		0.034	0.003	0.037	0.084
Bacillus subtilis		<0.001	<0.001	0.002	<0.001
Interaction		0.015	<0.001	0.016	0.083

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

CFU/kg B. subtilis and 25 mg or 45 mg/kg Zn showed the greatest values for ether extract, crude protein, and phosphorus ($P < 0.05$). The Zn level and its interaction with *B. subtilis* did not affect the retention of Zn ($P > 0.05$), while supplementation of *B. subtilis* at 5×10^9 CFU/kg increased Zn digestion compared with *B. subtilis* supplementation at 2.5×10^9 CFU/kg ($P < 0.05$). Supplementations of *B. subtilis* and Zn and their interaction had no significant effects on apparent digestion of crude fiber and the retention of calcium ($P > 0.05$).

Intestinal Histology

Significant interactions between Zn and *B. subtilis* were observed on the VL and VL/CD of both the jejunum and ileum, and the CD of the ileum ($P < 0.01$; Table 5), but not on CD of the jejunum ($P > 0.05$). The goose breeders fed the diets supplemented with 45 mg/kg Zn and 5.0×10^9 CFU/kg had the greatest values of VL and VL/CD in both the jejunum and ileum. The goose breeders fed the diet supplemented with 25 mg/kg Zn and 5.0×10^9 CFU/kg had the lowest value of CD in the ileum. Compared with *B. subtilis* supplementation at 2.5×10^9 CFU/kg, supplementation of *B. subtilis* at 5.0×10^9 CFU/kg significantly increased the CD depth of the jejunum (135.86 μm vs. 107.26 μm, $P < 0.01$).

Antioxidant Parameters in Serum

There were no significant interactions between *B. subtilis* and Zn on T-AOC and T-SOD in serum ($P > 0.05$;

Table 3. Effects of Zn and *Bacillus subtilis* supplementations on egg quality of goose breeders.[1]

Treatment						
Bacillus subtilis (CFU/kg)	Zn (mg/kg)	Eggshell strength (N)	Eggshell thickness (mm)	Albumen height (mm)	Yolk color	Haugh unit
5×10^9	25	5.04	0.47	16.30	3.13[ab]	120.05
	45	5.04	0.48	16.38	3.53[a]	119.48
	65	4.92	0.42	14.38	2.48[c]	114.10
2.5×10^9	25	4.97	0.42	13.93	2.40[c]	110.78
	45	5.03	0.45	15.63	2.68[bc]	116.48
	65	5.03	0.42	15.13	2.63[bc]	111.50
	Pooled SEM	0.06	0.02	0.96	0.17	4.83
Main effect						
	25	5.01	0.44	15.11	2.76	115.41
	45	5.04	0.47	16.00	3.10	117.98
	65	4.97	0.42	14.75	2.55	112.80
	Pooled SEM	0.04	0.01	0.68	0.12	3.42
5.0×10^9		5.00	0.46	15.68	3.04	117.88
2.5×10^9		5.01	0.43	14.89	2.57	112.92
	Pooled SEM	0.04	0.01	0.56	0.10	2.79
P-value						
Zn		0.582	0.071	0.427	0.016	0.573
Bacillus subtilis		0.895	0.088	0.328	0.003	0.225
Interaction		0.355	0.380	0.293	0.018	0.744

[a–c]Means with different superscripts within the same column differ significantly ($P < 0.05$).
[1]Data represent the means of 4 replicates, with 3 eggs per replicate.

Table 4. Effects of Zn and *Bacillus subtilis* supplementations on nutrient digestion and retention of goose breeders.[1]

Treatment *Bacillus subtilis* (CFU/kg)	Zn (mg/kg)	Crude fiber (%)	Ether extract (%)	Crude protein (%)	Calcium (%)	Zn (%)	Phosphorus (%)
5×10^9	25	23.30	77.46^{ab}	58.32^{ab}	64.57	49.66	49.68^{ab}
	45	25.42	80.29^{a}	59.32^{a}	65.32	53.26	55.93^{a}
	65	21.34	74.97^{bc}	55.70^{c}	58.68	41.99	44.99^{b}
2.5×10^9	25	21.11	73.70^{c}	55.22^{c}	57.05	39.24	44.32^{b}
	45	21.94	77.54^{ab}	56.36^{bc}	59.48	42.68	46.83^{b}
	65	21.99	77.44^{ab}	56.60^{bc}	62.89	44.89	49.49^{ab}
	Pooled SEM	1.05	1.07	0.82	4.20	3.10	2.13
Main effect							
	25	22.21	75.58	56.77	60.81	44.45	47.00
	45	23.68	78.92	57.84	62.4	47.97	51.38
	65	21.66	76.20	56.15	60.79	43.44	47.24
	Pooled SEM	0.74	0.76	0.58	2.97	2.19	1.51
5.0×10^9		23.35	77.57	57.78	62.86	48.30^{a}	50.20
2.5×10^9		21.68	76.22	56.06	59.80	42.27^{b}	46.88
	Pooled SEM	0.61	0.62	0.47	2.43	1.79	1.23
P-value							
Zn		0.167	0.014	0.143	0.908	0.330	0.097
Bacillus subtilis		0.067	0.141	0.019	0.385	0.028	0.073
Interaction		0.160	0.020	0.041	0.342	0.068	0.014

$^{a-c}$Means with different superscripts within the same column differ significantly ($P < 0.05$).
[1]Data represent the means of 4 replicates, with 1 female goose per replicate.

Table 6). Supplementation of *B. subtilis* at 5.0×10^9 CFU/kg increased T-AOC compared with *B. subtilis* supplementation at 2.5×10^9 CFU/kg (0.53 vs. 0.41 U/mL, $P < 0.05$), whereas it had no significant effect on T-SOD ($P > 0.05$). Supplementation of Zn had no significant effect on T-AOC ($P > 0.05$). But, a greater T-SOD was observed in birds supplemented with 45 mg/kg Zn compared with 25 mg and 65 mg/kg Zn ($P < 0.05$).

DISCUSSION

The positive effects of Zn and *B. subtilis* have been reported in poultry breeders and laying hens (Maria et al., 2018; Mayer et al., 2019; Neijat et al., 2019; Zhang et al., 2020), but little information is available on their combined use on reproductive performance of goose breeder. It was speculated that dietary probiotic may offer benefits to the digestion and absorption of mineral elements by improving the intestinal morphology (Neijat et al., 2019). In the present study, interactive effects between dietary Zn and *B. subtilis* was observed on the reproductive performance of goose breeder, and the groups fed with 5×10^9 CFU/kg *B. subtilis* and 25 mg or 45 mg/kg Zn had the greatest egg laying rate, fertility and hatchability rate. A previous study in our laboratory recommended with no probiotic supplementation, 65 to 70 mg/kg dietary Zn for improving the reproduction performance of laying goose breeder without probiotic supplementation (Shi et al., 2019). Therefore, we infer that 5×10^9 CFU/kg *B. subtilis* supplementation can reduce the dietary level of inorganic Zn by 30 to 65% in laying goose breeder.

Table 5. Effect of Zn and *Bacillus subtilis* supplementations on intestinal morphology of goose breeders.[1]

Treatment *Bacillus subtilis* (CFU/kg)	Zn (mg/kg)	Jejunum CD (μm)	Jejunum VL (μm)	Jejunum VL/CD	Ileum CD (μm)	Ileum VL (μm)	Ileum VL/CD
5.0×10^9	25	127.63	665.94^{b}	4.99^{ab}	72.79^{c}	385.83^{cd}	5.46^{ab}
	45	146.82	744.49^{a}	5.18^{a}	97.41^{bc}	576.35^{a}	6.23^{a}
	65	133.13	413.43^{c}	3.47^{c}	170.97^{a}	421.9^{bc}	2.47^{d}
2.5×10^9	25	119.77	406.38^{c}	3.36^{c}	120.17^{b}	379.39^{cd}	2.90^{cd}
	45	106.72	429.35^{c}	3.97^{bc}	85.91^{c}	311.88^{d}	3.58^{bcd}
	65	95.28	437.56^{c}	4.74^{ab}	123.26^{b}	495.94^{ab}	5.17^{abc}
	Pooled SEM	9.52	19.00	0.35	11.45	31.95	0.74
Main effect							
	25	123.70	536.16	4.18	96.48	382.61	4.18
	45	126.77	586.92	4.57	91.66	444.11	4.91
	65	114.21	425.50	4.11	147.11	458.92	3.82
	Pooled SEM	6.73	13.43	0.25	8.10	22.59	0.52
5.0×10^9		135.86^{a}	607.96	4.55	113.72	461.36	4.72
2.5×10^9		107.26^{b}	424.43	4.03	109.78	395.73	3.89
	Pooled SEM	5.50	10.97	0.20	6.61	18.44	0.43
P-value							
Zn		0.407	<0.001	0.371	<0.001	0.064	0.349
Bacillus subtilis		<0.001	<0.001	0.083	0.678	0.022	0.185
Interaction		0.196	<0.001	<0.001	<0.001	<0.001	0.003

$^{a-d}$Means with different superscripts within the same column differ significantly ($P < 0.05$).
[1]Data represent the means of 4 replicates, with 1 goose per replicate.Abbreviations: CD, crypt depth; VL, villus length; VL/CD, villus length/crypt depth.

Table 6. Effects of Zn and *Bacillus subtilis* supplementations on serum antioxidant capacity of goose breeders.[1]

Treatment *Bacillus subtilis* (CFU/kg)	Zn (mg/kg)	T-AOC (U /mL)	T-SOD (U /mL)
5×10^9	25	0.55	285.56
	45	0.64	335.56
	65	0.39	217.16
2.5×10^9	25	0.30	190.73
	45	0.48	287.02
	65	0.44	249.66
	Pooled SEM	0.08	35.52
Main effect			
	25	0.42	238.14[b]
	45	0.56	311.29[a]
	65	0.42	233.41[b]
	Pooled SEM	0.05	25.11
5.0×10^9		0.53[a]	279.43
2.5×10^9		0.41[b]	242.47
	Pooled SEM	0.04	20.51
P-value			
Zn		0.070	0.028
Bacillus subtilis		0.030	0.142
Interaction		0.080	0.120

Abbreviations: T-SOD, total superoxide dismutase; T-AOC, total antioxidant capacity.

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

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

In the present study, we revealed that supplementation with 45 mg/kg Zn and 5×10^9 CFU/kg *B. subtilis* increased the yolk color in goose breeders, indicating that the appropriate level of Zn and *B. subtilis* functioned synergistically on the pigment deposition in the yolk. Similarly, Kozłowski (2015) documented that inclusion of *B. subtilis* in the diets for laying hens resulted in greater yolk color score. The studies on the effects of dietary Zn supplementation on yolk color in laying poultry have been controversial. Broiler breeder hens fed the diet or supplemented with organic Zn had greater values of yolk color than those hens fed the diets without or with inorganic Zn (Li et al., 2015). In contrast, Zhang et al. (2020) and Liao et al. (2018) reported no effect on yolk color by dietary Zn supplementation in laying hens and duck breeders. A further study will be required to confirm the effect of Zn and *B. subtilis* on the yolk color.

The improvement in the reproduction performance of goose breeders may be attributed to the increased nutrient utilization. In the present study, supplementation with 5×10^9 CFU/kg *B. subtilis* and 25 mg or 45 mg/kg Zn improved the retention of ether extract, crude protein, and phosphorus. Similar results have also been observed in meat-type goose in our laboratory (Ke et al., 2018). Probiotic bacteria can increase the fermentation rate in the intestine, leading to the increased production of short-chain fatty acids and various enzymes such as protease, amylase, and lipase, which could further enhance the solubility of dietary nutrients (Santoso et al., 1995; Maria et al., 2018). In addition, we also found that supplementation with 5×10^9 CFU/kg *B. subtilis* increased the apparent digestion of Zn. Zn has a useful role in preventing oxidative damage in pancreatic tissue and activates pancreatic secretions of digestive enzymes and consequently stimulates the digestibility of nutrients (Sahin et al., 2005; Sahin et al., 2009). Our results suggested that the combined use of dietary Zn and *B. subtilis* can improve the retention of nutrients in geese and reduce pollution by decreasing the excretion of nitrogen, Zn, and phosphorus.

The small intestine is the main site for nutrient digestion and absorption in goose. The VL, CD and their ratio as the main indices of intestinal morphology, reflects the function, and health of the intestine. The increased VL and decreased CD are often associated with enhanced digestion and absorption of nutrients (De Grande et al., 2020). The positive effects of Zn and probiotics supplementation on intestinal morphology have been reported in broiler chickens (Bai et al., 2018; Wang et al., 2018; De Grande et al., 2020). In the present study, goose fed with 5×10^9 CFU/kg *B. subtilis* and 25 mg or 45 mg/kg Zn showed the greatest VL and VL/CD in both the jejunum and ileum, which could partly explain the improvement of the reproduction performance and nutrient digestion and retention in those birds. Similar results have been reported in broiler chickens: the dietary supplementation of Zn and probiotic either alone or in combination increased the VL in the duodenum (Shah et al., 2019), dietary *B. subtilis* improved the VL and CD of small intestine (Bai et al., 2018), adding 90 mg/kg Zn-Gly to the diet markedly decreased CD of the jejunum and ileum and increased VL of the jejunum and duodenum (Ma et al., 2011). The precise mechanisms through which probiotics improve the intestinal morphology are not clearly documented. However, it has been speculated that the increased volatile fatty acids production by dietary probiotic supplementation may nourish the intestinal villi and increase the proliferation of crypt cells and the turnover rate of epithelial cells.

Zinc plays a vital role in protecting the body from free radical and oxidative stress, by enhancing the activity of SOD (Bray and Bettger, 1990; Coudray et al., 1992). The increased antioxidant capacity by Zn supplementation has been observed in broilers, broiler breeders and turkeys (Akhavan-Salamata and Ghasemib, 2019; Huang et al., 2019; Jankowski et al., 2019). The current study found that goose breeders fed the diets supplemented with 45 mg/kg Zn showed greater T-SOD than the other groups. Our results are consistent with earlier studies in laying duck breeder that dietary Zn supplementation increases plasma T-SOD quadratically (Zhang et al., 2020). The T-AOC is an integrative parameter reflecting the antioxidant capacity of both enzymatic and non-enzymatic defense systems in serum and body fluids (Birben et al., 2012). The addition of *B. subtilis* has been reported to play a positive effect on antioxidant activity of broilers (Bai et al., 2018; Xu et al., 2021). Here, we found that 5×10^9 CFU/kg *B. subtilis* supplementation increased the T-AOC in serum of laying geese breeders, in agreement with our previous study (Wang et al., 2020).

In conclusion, our results demonstrate that dietary Zn and *B. subtilis* supplementations has positive effects on the reproductive performance, egg quality, antioxidant, nutrient digestion and absorption and intestine health of laying geese breeder. For the present study, dietary inclusion of 5×10^9 CFU/kg *B. subtilis* and 45 mg/kg inorganic Zn is the optimal combination for improving the reproductive performance of geese breeders.

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DISCLOSURES

The authors declare that they have no conflict of interest.

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