Effects of dietary *Enteromorpha* powder supplementation on productive performance, egg quality, and antioxidant performance during the late laying period in Zi geese

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ABSTRACT This study investigated the effects of dietary *Enteromorpha* powder supplementation on the productive performance, egg quality, and antioxidant performance of Zi geese during the late laying period. Three hundred twelve Zi geese (1 yr old) were randomly allocated into 2 cohorts to form a control group and an experimental group (with each cohort including 6 replicates and 21 female geese and 5 male geese in each replicate). The control group was fed a basal diet, and the experimental group was fed a diet containing 3% *Enteromorpha* powder. The data showed that *Enteromorpha* powder supplementation significantly improved egg production, laying rate, average daily egg weight ($P < 0.01$), and egg yolk color ($P < 0.05$). Supplementation decreased the ADFI and feed conversion rate ($P < 0.01$). Compared with the control group, glutathione peroxidase (**GSH-Px**) activity was significantly higher in serum and ovary tissue ($P < 0.05$), but GSH-Px activity was lower in liver tissue ($P < 0.01$). Malondialdehyde was reduced in liver and ovary tissue ($P < 0.05$) in the *Enteromorpha* powder supplementation group. Meanwhile, the expression of the *CAT* gene was significantly upregulated in the liver ($P < 0.01$) in the *Enteromorpha* group. These results indicate that dietary *Enteromorpha* powder supplementation improved productive performance and reduced the level of lipid peroxidation in Zi geese during the late laying period.

Key words: *Enteromorpha* powder, Zi goose, productive performance, egg quality, antioxidant

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INTRODUCTION

Enteromorpha is a large green seaweed (Blomster and Fewer, 2002). Recently, *Enteromorpha* has disrupted the aquaculture industry and tourism along the coast of China because of massive proliferation caused by sea pollution. Notwithstanding the disruption of local industries, *Enteromorpha* is an excellent feed for animals. The amino acids in *Enteromorpha* are balanced and thus easy for animals to digest and absorb. *Enteromorpha* is also rich in minerals, vitamin A, and vitamin C. Similar to other seaweeds, *Enteromorpha* contains a large number of active substances, such as seaweed polysaccharides and polyunsaturated fats, which have proven to be antiviral, antitumor, antioxidant, and hypolipidemic and can enhance immunity and other physiological activities (Damonte et al., 2004; Yuan and Walsh, 2006; Cho et al., 2011; Kim et al., 2011; Pereira et al., 2012). Late in the laying period, ovaries begin to be attacked by reactive oxygen species and gradually atrophy, which further leads to a decline in the number and quality of follicles (Garg and Sinclair, 2015). Tarin (1996) has shown that oxidative stress is the leading cause of ovarian failure. Furthermore, various studies demonstrate that with the decline in ovarian function, the body's antioxidant activity is weakened, as is the reactive oxygen species clearance efficiency (Carbone et al. 2003). Liu et al. (2018) observed that grape seed proanthocyanidin extract prevented ovarian aging through inhibition of oxidative stress in hens. Therefore, supplementation with antioxidants in the feed should increase systemic antioxidant levels and delay the aging of

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ovaries, which is of great significance for improving egg production (**EP**) of geese during the late laying period.

There are no reports of *Enteromorpha* application as a feed ingredient in laying geese. In this study, 3% *Enteromorpha* powder was used to replace some of the other ingredients in the basal diet to investigate how *Enteromorpha* affects productive performance and internal antioxidant performance during the late laying period in Zi geese. This thesis will provide a theoretical basis for improving EP in poultry.

MATERIALS AND METHODS

This experiment was conducted in accordance with the Chinese guidelines for animal welfare and with the animal welfare standards of the College of Animal Science and Technology, Northeast Agricultural University.

Geese Experiment Design, Diets, Feeding, and Management

A total of 312 Zi geese (1 yr old), with similar health status and body weight, evaluated using an electronic scale (ACS-809; Yongkang Huaying Weighing Apparatus Co., Ltd., Yongkang, China) with accuracy of 1 g, were randomly distributed into 2 groups (the control group and experimental group) with 6 replicates per group and 21 female geese and 5 male geese per replicate. The control group was fed with basal diet, and the experimental group was fed with a diet containing 3% *Enteromorpha* powder. *Enteromorpha* powder was purchased from Zhongtaihe Biotechnology Co., Ltd., Qingdao, China. *Enteromorpha* powder contains 6.64% crude protein, 6.00% crude fiber, 0.10% Met, and 0.18% Lys. The dietary composition and nutritional levels are shown in Table 1.

The geese were housed outside with a shade shelter that provided a stocking density of 5.2 birds/m^2 and adopted natural light. The air temperature during the test period was 17 to 34°C, and the humidity was 20% to 80%; the length of daylight was 14.5 to 15.5 h. At 6 o'clock every morning, all geese were allowed access to water and feed ad libitum. The goose eggs were collected at 9 am and 3 pm. The feces were cleaned from the pen every 3-4 days. The pretrial period was 1 week, and the trial lasted 8 weeks in total.

Productive Performance

The total number of eggs, egg weight, and unqualified eggs (broken, oversized, too small, or soft-shell eggs) in each replicate were recorded every day. Egg weight was measured with an electronic scale (LT201C; Changshu Tianliang Instrument Co., Ltd., Changshu, China) with accuracy of 0.1 g. EP, laying rate (**LR**), qualified egg rate, fertility rate (**FR**), and average daily egg weight (**ADEW**) were calculated from the records. The FR = (fertilized eggs/hatching eggs) × 100. The ADFI = (weekly added feed amount–weekly remaining feed amount)/7. The feed conversion ratio (**FCR**) = ADFI/ADEW.

Table 1. The compositions of the diet and the nutritional level (air-dried basis, %).

Ingredient	Control group	Experimental group
Corn	39.60	39.60
Corn gluten feed	21.00	18.40
Corn germ meal	20.00	19.60
Corn oil	0.50	0.50
Soybean meal	10.00	10.00
Enteromorpha powder	0.00	3.00
Limestone	3.50	3.50
Dicalcium phosphate	0.90	0.90
Salt	0.35	0.35
DL-methionine	0.15	0.15
Lysine	0.01	0.01
Choline chloride	0.08	0.08
Premix	0.39	0.39
Zeolite powder	3.52	3.52
Total	100.00	100.00
Nutrients		
ME (MJ/kg)	10.04	10.04
CP	15.59	15.41
Met	0.38	0.38
Met + Cys	0.50	0.49
Lys	0.64	0.63
Ca	1.64	1.68
Total phosphorus	0.58	0.56
Available phosphorus	0.26	0.26

Each kilogram of diet contains the followings: vitamin A, 15,000 IU; vitamin D3, 5,300 IU; vitamin E, 100 mg; vitamin K, 4 mg; vitamin B1, 2 mg; vitamin B2, 10 mg; vitamin B6, 10 mg; vitamin B12, 0.1 mg; niacin, 100 mg; pantothenic acid, 50 mg; folic acid, 2 mg; biotin, 0.3 mg; Fe, 120 mg; Cu, 20 mg; Zn, 100 mg; Mn, 600 mg; I, 3 mg; and Se, 0.5 mg.

Egg Quality

On the 42nd and 56th days of the experiment, 3 eggs were collected from each replicate and stored in a 4°C refrigerator (SC-320D; Haier Smart Home Co., Ltd., Qingdao, China), and egg quality was measured within 24 h. The average egg weight was measured using an electronic scale (LT201C; Changshu Tianliang Instrument Co., Ltd.). Transverse and longitudinal diameters of the eggs were measured using a vernier caliper (G-CRAFT; Jinhua Shijiang Tools Co., Ltd., Jinhua, China). Eggshell strength was evaluated using an eggshell strength meter (NFN388; Fujihira Industry Co. Ltd., Tokyo, Japan). Eggshell thickness was calculated as the mean value of measurements acquired from 3 locations on the shell (the blunt end, middle, and sharp end) using an eggshell thickness gauge (NFN380; Fujihira Industry Co., Ltd.). Albumen height was measured using an egg quality gauge and egg quality measurement stand (NFN381 and NFN382; Fujihira Industry Co. Ltd.). Egg yolk was separated from the egg white, and then, the egg yolk color was determined by comparison with a color fan (Robotmation Co., Ltd., Tokyo, Japan). In addition, the Haugh unit score was calculated from the albumen height and egg weight. The egg shape index = egg longitudinal diameter/egg transverse diameter. The Haugh unit score = $100 \cdot \log(\text{albumen height} - 1.7X \text{ egg weight}^{0.37} + 7.57)$ (Onbasilar et al., 2011).

Table 2. Fluorescent quantitative primer information.

Genes	Primer sequences (5′-3′)	Product length/bp
SOD1	F:CACCTGCTGTAACCATTCTTAGT R:GGCTCCTCATCTTCCAAACC	137
GSH-Px4	F:AGATTAAGGCGTTTGCTGAGAA R:CGGTTGATGAGGAACTTAGTGAA	172
CAT	F:GCCTGTTACTTCTTCCTCTTCC R:ATCATCATCCTCCTTCCAATCTG	157
GAPDH	F:TAGTGAAGGCTGCTGCTGAT R:AGGTGGAGGAATGGCTGTC	102

Abbreviations: CAT, catalase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH-Px4, glutathione peroxidase 4; SOD, superoxide dismutase.

Antioxidant Analysis

At the end of week 6, and 12 h after feed withdrawal, 1 female goose of similar body condition was taken from each replicate. Five milliliters of blood was collected from the axillary vein. Serum samples were centrifuged at 4,000 r/min for 10 min and then analyzed. At the end of the trial (week 8), 1 female goose from each replicate was slaughtered. The liver and ovarian bases were collected from each goose. Homogenates were prepared by adding physiological saline to the tissue. Superoxide dismutase (**SOD**), glutathione peroxidase (**GSH-Px**), catalase (**CAT**), and malondialdehyde (**MDA**) were all measured using commercial kits bought from Nanjing Jiancheng Bioengineering Institute, Nanjing, China.

Quantification of SOD1, GSH-Px4, and CAT Genes With Real-Time PCR

Fifty milligrams of each collected tissue sample was thoroughly ground in liquid nitrogen and transferred into a 1.5-ml EP tube for further analysis. Total RNA was extracted with an RNA extraction kit (GENEray, GK3006), and levels of relative expression of SOD1, GSH-Px4, and CAT genes were determined with real-time PCR. Primers for SOD1, GSH-Px4, and GAT were selected according to the sequences of geese registered in NCBI and designed by using Beacon Designer 7. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. The primer sequences are shown in Table 2. The total volume of the real-time PCR system was 10 μl, and the reaction system was as follows: SYBR Green Mix 4.4 μl, upstream and downstream primers 0.3 μl each, and cDNA 5 μl. The real-time PCR procedure was as follows: 95°C for 10 min, 1 cycle, 95°C for 10 s, 60°C for 34 s, and 40 cycles. The Ct values of the target genes and the internal reference genes were measured, and the relative expression levels of the antioxidant genes were calculated by the $2^{-\Delta\Delta}{}_{Ct}$ method.

Statistical Analysis

All data were analyzed with two-tailed Student's *t*-tests using SPSS 22.0 (SPSS Inc., Chicago, IL). The analysis results were expressed as arithmetic mean and standard error of mean. Differences were considered to be significant at $P < 0.05$ and highly significant at $P < 0.01$.

RESULTS

Production Performance

As shown in Table 3, dietary *Enteromorpha* powder supplementation significantly increased EP, LR, and ADEW ($P < 0.01$). However, no effects on the qualified egg rate or FR were found from *Enteromorpha* supplementation ($P > 0.05$). The results also showed that the ADFI and FCR were significantly lower with *Enteromorpha* powder than in the control group ($P < 0.01$).

Egg Quality

Egg quality data are summarized in Table 4. At the end of week 6, the egg yolk color in the experimental group was significantly improved ($P < 0.05$). However, the experimental diet had no significant effects on average egg weight, egg shape index, eggshell strength, eggshell thickness, albumen height, or the Haugh unit score ($P > 0.05$). At the end of week 8, no indicators of egg quality were significantly different between treatment and control groups ($P > 0.05$).

Antioxidant Analysis

The effects of dietary *Enteromorpha* powder on antioxidant activity are shown in Table 5. In contrast to the control group, *Enteromorpha* powder significantly increased GSH-Px activity in serum ($P < 0.01$), whereas SOD, CAT, and MDA did not exhibit obvious differences between the treatments ($P > 0.05$). Dietary *Enteromorpha* powder supplementation significantly reduced GSH-Px activity and MDA levels in liver tissue ($P < 0.01$ and $P < 0.05$). In agreement with data from serum, SOD and CAT activity levels were not different between the 2 groups ($P > 0.05$). Addition of *Enteromorpha* powder to the diets significantly increased GSH-Px activity and decreased MDA levels in ovary tissues ($P < 0.05$). Compared with the control diet, treatment did not significantly affect SOD or CAT activity ($P > 0.05$).

Table 3. The effects of dietary *Enteromorpha* powder on productive performance in Zi geese.[1]

Item	Control group	Experimental group	SEM	*P*-value
EP (eggs/week/replicate)	6.57[B]	14.60[A]	0.51	<0.01
LR (%)	4.51[B]	10.11[A]	0.35	<0.01
QER (%)	90.86	91.58	2.15	0.714
FR (%)	75.93	75.31	2.42	0.804
ADFI (g)	196.16[A]	186.95[B]	2.85	<0.01
ADEW(g)	5.45[B]	12.54[A]	0.43	<0.01
FCR	36.23[A]	14.97[B]	1.49	<0.01

Abbreviations: ADEW, average daily egg weight; ADFI, average daily feed intake; EP, egg production; FCR, feed conversion ratio; FR, fertility rate; LR, laying rate; QER, qualified egg rate.

[A, B]Means within a row with no common superscripts indicate a highly significant difference ($P < 0.01$).

[1]Goup means were represented as the mean of the corresponding data from 6 replicates (26 birds per replicate).

Analysis of Expression of SOD1, GSH-Px4, and CAT Genes

The data for liver and ovary antioxidant gene expression are shown in Table 6. No significant differences were observed for the expression of SOD1 or GSH-Px4 in liver tissues between the 2 groups ($P > 0.05$). However, expression levels of CAT were significantly improved with *Enteromorpha* supplementation ($P < 0.01$). No differences were found between the 2 groups in expression levels of SOD1, GSH-Px4, or CAT in ovary tissues ($P > 0.05$).

DISCUSSION

In this study, we found that EP, LR, and ADEW were improved and the FCR was reduced by the *Enteromorpha* powder; however, it had no effect on the FR. These results indicate an improvement in the productive performance of Zi geese. Skřivan et al. (2006) found that laying hens fed diets with 1.2 g/kg Se-enriched *Chlorella* exhibited a considerably higher egg weight and LR than those in the control group. Abudaboset et al. (2013) observed that substituting 3.0% of corn with seaweed did not significantly affect the ADFI in broiler chickens. However, our study showed that the ADFI was reduced by supplementation with *Enteromorpha* in Zi geese, possibly because geese have a stronger ability to digest the crude fiber. The goose has a developed cecum, which is rich in microorganisms. The microorganisms may catabolize the crude fiber into short-chain fatty acids to provide energy for the body. In addition, the crude fiber exerts a strong hydraulic force, which could increase the volume of the chyme and facilitate keeping the gut full (Jamroz et al., 1992; Marounek et al., 1999).

Many studies have shown that adding seaweed to poultry diets can improve egg quality. Zahroojian et al. (2011) reported that after 1.5-2.5% *Spirulina* was added to the laying hen diet, the egg yolk color improved significantly compared with that in the control group. The improvement in egg yolk color was partly due to the change in carotenoid composition (Jensen, 1963). Based on the results of our research, we drew a similar conclusion. There were no significant differences in other indicators between the *Enteromorpha* supplementation group and the control group. Previous studies have shown that seaweed contains significant quantities of protein, crude fiber, lipids, minerals, and vitamins

Table 4. The effects of dietary *Enteromorpha* powder on egg quality in Zi geese.[1]

Item	Control group	Experimental group	SEM	*P*-value
Wk 6				
Average egg weight (g)	125.56	128.59	4.71	0.534
Egg shape index	1.46	1.45	0.01	0.535
Egg yolk color	5.06[b]	5.72[a]	0.27	0.029
Eggshell strength (N)	67.17	69.66	3.12	0.444
Eggshell thickness(mm)	0.54	0.55	0.01	0.668
Albumen height (mm)	6.32	6.17	0.57	0.806
Haugh unit	54.65	51.64	6.64	0.659
Wk 8				
Average egg weight (g)	123.98	126.78	3.57	0.450
Egg shape index	1.48	1.46	0.01	0.792
Egg yolk color	4.50	4.67	0.23	0.488
Eggshell strength (N)	68.99	63.36	6.09	0.377
Eggshell thickness (mm)	0.53	0.51	0.01	0.209
Albumen height (mm)	5.36	5.36	0.44	0.989
Haugh unit	41.20	39.88	6.97	0.853

[a, b]Means with a row with no common superscripts differ significantly ($P < 0.05$).

[1]Goup means were represented as the mean of the corresponding data from 6 replicates (3 eggs per replicate).

Table 5. The effects of dietary *Enteromorpha* powder on antioxidant analysis in Zi geese.[1]

Item	Control group	Experimental group	SEM	*P*-value
Serum				
SOD (U/ml)	126.88	123.87	2.46	0.247
GSH-Px (U/mgprot)	267.04^{B}	295.12^{A}	8.42	<0.01
CAT (U/mL)	2.11	2.08	0.08	0.693
MDA (nmol/ml)	4.70	4.57	0.12	0.732
Liver				
SOD (U/mgprot)	159.65	159.37	10.40	0.979
GSH-Px (U/mgprot)	192.30^{A}	146.88^{B}	13.81	<0.01
CAT (U/mgprot)	2.40	2.44	0.20	0.869
MDA (nmol/mgprot)	2.43^{a}	1.96^{b}	0.18	0.026
Ovary				
SOD (U/mgprot)	195.98	217.01	15.20	0.197
GSH-Px (U/mgprot)	18.43^{b}	23.76^{a}	2.34	0.046
CAT (U/mgprot)	0.58	0.62	0.09	0.656
MDA (nmol/mgprot)	1.96^{a}	1.48^{b}	0.16	0.012

Abbreviations: CAT, catalase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase.

$^{a, b}$Means with a row with no common superscripts differ significantly ($P < 0.05$).

$^{A, B}$Means within a row with no common superscripts indicate a highly significant difference ($P < 0.01$).

[1]Group means were represented as the mean of the corresponding data from 6 replicates (2 birds per replicate for serum samples, 1 bird per replicate for liver and ovary samples).

(Norziah and Ching, 2000; Wong and Cheung, 2000). In addition, Fleurence et al. (1999) demonstrated that *Ulva armoricana* also contains high levels of essential amino acids. In addition, many active substances, such as acidic polysaccharides, polyunsaturated fatty acids, carotenoids, and other trace elements, exist in seaweed (Wang et al., 2013; Miedico et al., 2016; Ren et al., 2018). These active substances have physiological functions, such as improving immunity, antioxidation activity, and anticancer activity. Late in the laying period, the ovary gradually shrinks due to oxidative stress, which leads to a decline in the EP rate. Therefore, we speculated that the active substances in *Enteromorpha* powder could increase antioxidant capacity in geese.

The results of this study showed that supplying *Enteromorpha* powder in diets significantly increased GSH-Px activity in serum and ovary tissue but decreased GSH-Px activity in liver tissue. GSH-Px is an important peroxide-degrading enzyme whose main function is to remove the peroxide and hydroxyl radicals produced by metabolic processes, protect cell membranes and structural integrity, and contribute to the maintenance of the oxidation/antioxidant balance (Richter, 1987; Chi et al., 2017). Peng et al. (2009) suggested that *Chlorella* extract could increase GSH-Px activity, reduce the oxidative stress caused by carbon tetrachloride, and reduce the levels of liver MDA in rats. SOD, which directly participates in antioxidant function by scavenging O^{-}_{2} radicals, is one of the most important antioxidant enzymes in organisms (Chen et al., 2013). Previous studies have shown that SOD activity can be significantly increased in purified sulfated polysaccharides extracted from seaweed compared with that in ascorbic acid in vitro (Hoang et al., 2015). Zhang et al. (2003) reported that in aging mice, injection of 200 or 400 mg/kg *Porphyra haitanensis* polysaccharide can increase liver GSH-Px and SOD activities. Wang et al. (2015) have shown that sulfated polysaccharides show a better protective effect against H_2O_2-induced oxidative stress. CAT is an antioxidant enzyme that specifically removes H_2O_2 from tissues and catalyzes the transfer of electrons to decompose H_2O_2 into water and oxygen, thereby reducing oxidative stress (Schrader and Fahimi, 2006). Raghavendran et al. (2005) reported that *Sargassum polycystum* (brown alga) extract can increase CAT activity and reduce lipid peroxidation in rat liver tissue. We found that *Enteromorpha* powder did not increase GSH-Px, SOD, or CAT activities in goose liver tissue, possibly because of the antioxidant mechanism of *Enteromorpha* powder. *Enteromorpha* exerts its antioxidant effect through polysaccharides and polyunsaturated fatty acids, which are rich in *Enteromorpha*, rather than through elevation of GSH-Px, SOD, and CAT activities in goose livers. Li et al. (2013) showed that high-molecular-weight polysaccharides had inhibitory effects on superoxide radicals, and low-molecular-weight polysaccharides had inhibitory effects on

Table 6. The effects of dietary *Enteromorpha* powder on SOD1, GSH-Px4, and CAT gene expression in Zi geese.[1]

Item	Control group	Experimental group	SEM	*P*-value
Liver				
SOD1	1.00	0.88	0.07	0.106
GSH-Px4	1.00	0.92	0.16	0.623
CAT	1.00^{B}	1.36^{A}	0.10	<0.01
Ovary				
SOD1	1.00	1.01	0.13	0.926
GSH-Px4	1.00	0.91	0.13	0.516
CAT	1.00	0.88	0.10	0.249

Abbreviations: CAT, catalase; GSH-Px4, glutathione peroxidase 4; SOD, superoxide dismutase 1.

$^{A, B}$Means within a row with no common superscripts indicate a highly significant difference ($P < 0.01$).

[1]Group means were represented as the mean of the corresponding data from 6 replicates (1 bird per replicate).

hydroxyl radicals at low concentrations. MDA is the product of lipid peroxidation; therefore, levels of MDA can be used to indicate the extent of lipid peroxidation mediated by oxygen free radicals (Janero, 1990; Mujahid et al., 2007). Our results showed that supplementation with *Enteromorpha* powder significantly reduced MDA in the liver and ovary, which also proved that *Enteromorpha* powder can reduce lipid peroxidation in the body, mainly by being oxidized.

This trial showed that, in the liver and ovary, SOD1and GSH-Px4 expression were not significantly different between the *Enteromorpha* powder supplementation group and the control group. The group receiving *Enteromorpha* powder showed an upregulation of CAT expression in the liver but no effect on CAT expression in ovary tissues. The expression of SOD1 showed a consistent trend among serum and liver and ovary tissues, indicating that the *Enteromorpha* powder had no significant effects on the regulation of SOD, while regulation of GSH-Px was only at the protein level, which may affect the gene over a longer time. Abril et al. (2014) showed that adding *Ulva linza* to high-fat and high-sugar diets for rats decreased liver GSH-Px activity. Similarly, no obvious effect of liver GSH-Px gene expression on the treated group was observed, agreeing with the findings from our experiment. Although the addition of *Enteromorpha* powder upregulated liver CAT activity, CAT activity did not increase in the ovary, possibly because organs may differ in sensitivity to the *Enteromorpha* powder. In addition, CAT activity and gene expression showed an inconsistent trend in the liver, which may be because genes are regulated by many factors during translation; therefore, the activity of CAT were perhaps affected not only by concentration but also by the presence of activators or inhibitors.

In summary, based on the above results, we concluded that dietary supplementation with *Enteromorpha* powder improved EP, LR, ADEW, and egg yolk color. In addition, *Enteromorpha* powder reduced ADFI and FCR, increased GSH-Px activity in serum and ovary tissues, and reduced MDA levels in liver and ovary tissues to exert an antioxidant effect during the late laying period of Zi geese.

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