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Ulvan extracted from green seaweeds as new natural additives in diets for laying hens

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Abstract

Ulvan extracted from the green seaweed *Ulva* was added in laying hen diets. The effect of ulvan on laying performance, egg quality, immunity function, and antioxidant capacity was evaluated using Hy-Line Brown hens. Six groups of birds ($n = 864$, 61 weeks old) were fed on the basal diet containing ulvan at 0, 0.05, 0.1, 0.5, 0.8, and 1% for 8 weeks. The results were compared with the control group. Ulvan at concentrations of 0.1 to 1% can significantly improve the egg production ($P < 0.05$), and higher concentrations (1%) increased the egg weight and decreased feed conversion ratio of hens ($P < 0.05$). Ulvan at higher concentrations (0.8%, 1%) also helped to improve the eggshell strength ($P < 0.05$). Ulvan at concentrations of 0.5 to 1% leads to a yolk color to red tendency, and ulvan at concentrations 0.05 to 1% can significantly decrease cholesterol levels of yolk ($P < 0.05$). Treatments with high levels (1%) of ulvan showed a positive effect on interleukin-6 as well as 0.8% of ulvan on interferon-γ ($P < 0.05$). There are significant interactions ($P < 0.05$) on the time × ulvan level on total antioxidative capacity, malondialdehyde, and superoxide dismutase levels of blood serum. These findings thus suggested that ulvan extract can be used as additives in diets for laying hens.

Keywords Ulvan · Laying hens · Laying performance · Egg quality · Immunity function · Antioxidant capacity

Introduction

In recent years, many kinds of poultry diseases have emerged. Farmers have to rely on antibiotic treatment against various diseases. The excessive and indiscriminate use of antibiotics drugs in the poultry industry has led to concerns such as development of antibiotic resistant strains of pathogens, high concentrations of antibiotic residues in meat or egg products, and undesirable changes in the microbial communities of animal intestinal tracts (Filazi et al. 2005; Shargh et al. 2012; Tellez et al. 2012). We need to find new alternatives to replace or reduce overuse of antibiotics in the breeding industry of poultry. Thus, can we find a kind of natural biological product which cannot only eliminate or prevent poultry disease but also improve the quality of food such as meat and eggs? With the continuous development of marine resources, countless species of algae with favorable biological activity have been reported to be acceptable for inclusion in diets for rats, broiler chickens, laying hens, swine, and other animals (Becker 2007; Kotrbáček et al. 2015). Kulshreshtha et al. (2014) reported that feed supplementation with red seaweeds affects performance, egg quality, and gut microbiota of laying hens. El-Deek and Brikaa (2009) also found that feeding seaweeds has led to an increase in the growth rate and nutrient uptake in chickens and ducks.

Algae contain abundant carbohydrate, fibers, protein, and a variety of vitamins. The cell walls of marine algae are rich in sulfated polysaccharides which are becoming more and more important in biochemical and medical fields (Zhang et al. 2010; Souza et al. 2012). Sulfated polysaccharides have a

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wide range of important biological activities such as immunomodulation, antiviral, anticoagulant, antioxidant, anticancer, antiallergy, and anti-inflammation (Liu et al. 2012; Ngo and Kim 2013; Shao et al. 2013). Sulfated polysaccharides extracted from marine algae include carrageenan from red algae, ulvan isolated from green algae, and fucoidan from brown algae (Cunha and Grenha 2016). Some significant evidences have indicated that the biological activities of polysaccharides will depend on their structural features, such as the degree of sulfation and distribution pattern of sulfate, molecular weight, branch structures, type of glycosidic linkages, and monosaccharide composition (Ferreira et al. 2015; He et al. 2016; Seedevi et al. 2017). The abundant, heavily sulfated ulvans are extracted from green seaweed of the *Ulva* sp. These algae are commonly found in coastline areas of the world. They grow abundantly in nutrient-enriched zones, float congregating along the coast, block waterways, and destroy the marine ecosystem, which becomes a serious threat to the coastal fishing industry and development of tourism. Accordingly, it will be of great practical implication for us to make waste profitable with *Ulva*. Ulvan consists of rhamnose, xylose, glucose, uronic acid, and sulfate (Aguilar-Briseno et al. 2015), which regulate immune functions (Geng et al. 2016) and act as antioxidant (Meenakshi et al. 2011) and antibacterial (Berri et al. 2016). Ulvan with high level of sulfated polysaccharide has also been found to have anticoagulant, antiviral, anti-inflammatory, and antihyperlipidemic activities in cells or rats (Freitas et al. 2015; Qi and Sheng 2015; Qi and Sun 2015; Synytsya et al. 2015; Araújo et al. 2016).

However, there are limited reports on the feeding values of the ulvan as supplementation in diets for poultry such as laying hens. So can ulvan be used as a new prophylactic for reducing the subsequent use of antibiotic treatment in poultry breeding industry? The objective of this study was to determine the effect on productive performance, egg quality, immunoregulation, and antioxidant for laying hens fed with different doses of ulvan extracted from green seaweeds.

Materials and methods

Extraction of ulvan

Green seaweed *Ulva* were collected in the coast of the Yellow Sea, Shandong Peninsula, East China (N 35.85–36.70°, E 120.05–121.20°, near Qingdao). The algae were washed with fresh water and ground into powder after drying at room temperature for 24 h. Dried powder was macerated in water (6:1 distilled water/algae powder, *w*/*w*) with 2% cellulase. The mixture was incubated at 55 °C for 3 h. Then, 1% hydrogen peroxide was added at the mixture for 6 h (pH 4.0). Finally, ulvan was generally evaporated to dryness.

Composition analysis

Ulvan in the present study was not completely purified. Total carbohydrate content was measured with the phenol-sulfuric method (Dubois et al. 1956) using glucose as standard. Uronic acid was determined with sulphuric acid-carbazole method with glucuronic acid as standard substance (Bitter and Muir 1962). Sulfate content was determined by $BaCl_2$-gelation method (Yuan 2015b). Protein content was measured with Folin-phenol method and the standard substance was bovine albumin (Lowry et al. 1951). The final composition of the crude ulvan includes glycosyl (50.77%), glucuronic acid (21.28%), sulfate (21.65%), protein (6.35%), and trace salts. Ulvan (typically 2 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 105 °C under nitrogen for 6 h. The monosaccharide hydrolysate was dried under a vacuum and then derivatized with 120 μL of PMP solution (0.5 M, in methanol) and 100 μL of 0.3 M NaOH at 70 °C for 1 h. The reaction was stopped by neutralization with 100 μL of 0.3 M HCl and extraction with CH_2Cl_2 (700 uL, three times). HPLC analyses were performed on an Agilent Zorbax XDB-C18 column (25 cm × 4.6 mm × 5um) at 35 °C with detection at UV 245 nm. The mobile phase was 0.05 M KH_2PO_4 (pH 6.7) with 83% (solvent A) and 17% (solvent B) acetonitrile in water (Strydom 1994). The contents of monosaccharides in the ulvan were calculated from the peak areas with rhamnose, glucose, xylose, galactose, arabinose, and mannose as standard substance (Fig. 1). The percentages were 49.52% rhamnose, 45.39% glucose, 2.88% xylose, 0.87% galactose, 0.91% arabinose, and 0.43% mannose.

Experimental birds and design

Hy-Line Brown hens ($n = 864$, 61 weeks old) (Animal Experimental Ethical Inspection) were randomly assigned into six groups. Diet 1 (D1) was fed with control diet containing basic fodder (Table 1); the other five groups (D2, D3, D4, D5, and D6) were fed with diets consisting of different concentrations of ulvan: 0.05% (D2), 0.1% (D3), 0.5% (D4), 0.8% (D5), and 1% (D6). Each treatment consisted of six replicates with eight cages and three hens per cage. The cages were made of galvanized metal wire (approximately 55 cm × 37 cm × 40 cm). A regime of 16 h light was provided and the temperature was set at 25 °C. All the layers were kept under the same management conditions throughout the experimental period of 8 weeks.

Collection and analyses

Eggs were collected daily. The egg numbers were recorded during 61 to 68 weeks of age. Egg production was calculated

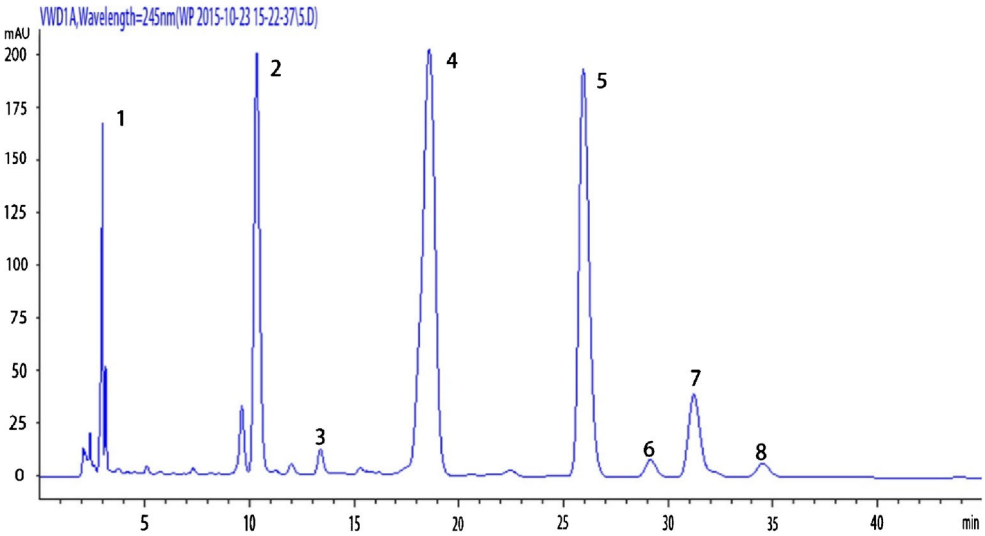

Fig. 1 Liquid chromatograph of ulvan monosaccharides. 1—impurities, 2—PMP, 3—mannose, 4—rhamnose, 5—glucose, 6—galactose, 7—xylose, and 8—arabinose

as number of eggs per day/number of hens. Feed intake was determined at the end of every 2 weeks of the trial. All eggs of every group during the last three consecutive days of the 2-week period were collected and egg weight was recorded, then average egg weight was obtained. Feed conversion ratio (FCR) was calculated as grams of feed per gram of egg.

Thirty eggs were randomly chosen in each treatment from the eggs laid during the last day of each 28-day period to determine the egg-shaped index using a vernier caliper; the egg-shaped index was computed as egg height (mm)/egg width (mm). Eggshell breaking strength was detected by an egg force reader (Orka Technology Ltd., Ramat Hasharon, Israel); thereafter, eggs were cracked, carefully separating the eggshell, and albumen height, Haugh unit (HU), and egg yolk color were measured using SONOVA egg analyzer (Orka Technology Ltd., Ramat Hasharon, Israel). The HU was calculated from the height of the inner thick albumen and the weight of an egg (Wang et al. 2015b). The yolk was separated from the egg, adding four times the volume by weight (g:mL) of anhydrous ethanol, mixed thoroughly for 2–3 min with a grinding rod, then transferred the solution into 1.5-mL Eppendorf tubes, and centrifuged at 3500 rpm for 10 min; egg cholesterol content was measured using the supernatant with the Cholesterol Reagent Kit provided by Nanjing Jiancheng Biology Engineering Institute.

At the middle (64 weeks of age) and the end (68 weeks of age) of the experiment, blood samples were taken by puncturing wing vein from one bird per replicate. The whole blood was put in a constant-temperature incubator at 37 °C for approximately 30 min and then centrifuged at 3500 rpm for 10 min to acquire the serum at room temperature. Serum samples were aspirated by pipette and stored in 1.5-mL Eppendorf tubes at − 80 °C. The serum on ice was unfreezed before analysis. To the immunity function of hens, individual serum samples were analyzed for IFN-γ (interferon-γ), IL-6 (interleukin-6), IgG (immunoglobulin G), and AVI-Ab (antibodies to avian influenza virus) by ELISA technique using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The plates were read in Synergy H1 Multi-Mode Reader (BioTek, USA). The activities of SOD (superoxide dismutase), MDA (malondialdehyde), and CAT (total antioxidative capacity) were assayed according to the commercial kit (Nanjing Jiancheng Bioengineering Institute) manufacturer's instructions.

Table 1 Composition and nutrient levels of experimental diets

Ingredients	Contents (%)	Nutrient levels	Contents
Corn	56.00	ME (J kg^{-1})	12.54
Soybean meal	31.48	CP	21.52
Maize protein powder	3.50	Ca	1.02
Soya-bean oil	3.30	AP	0.45
Fish meal	2.30	Lys	1.17
Limestone	1.30	Trp	0.24
$CaHPO_4$	1.50	Arg	1.41
NaCl	0.30	Met + Cys	0.83
Choline chloride	0.10		
Vitamin premix[1]	0.02		
Mineral premix[2]	0.20		
Total	100		

[1] Vitamin premix provided per kilogram of the diets: vitamin A, 12,500 IU (from retinyl acetate); vitamin D_3 (cholecalciferol), 3000 IU; vitamin E (from DL-α-tocopheryl acetate), 10 IU; vitamin K_3, 3 mg; vitamin B_1, 2 mg; vitamin B_2, 7.0 mg; vitamin B_6, 3.0 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 11 mg; nicotinic acid, 50 mg; folic acid, 1 mg; biotin, 0.05 mg

[2] Mineral premix provided per kilogram of the diets: Cu, 8 mg; Zn, 60 mg; Fe, 40 mg; Mn, 100 mg; I, 0.8 mg; Se, 0.15 mg

Statistical analyses

Data were subjected to ANOVA using SPSS software, version 22.0 (SPSS Inc., USA); Duncan's multiple-range test was carried out to detect differences among treatments. The effects of ulvan on immunity function and antioxidant capacity of laying hens were analyzed by ANOVA in 6 (ulvan level) × 2 (time) factorial arrangements of treatments. The results are presented as means ± SE. All differences were considered significant at $P < 0.05$.

Results

Effects of ulvan on laying performance of laying hens

The effect of ulvan dietary supplementation on egg production rate of laying hens during 61 to 68 weeks has been presented in Table 2, and there was a significant ($P < 0.05$) difference among different diets (D1 to D6) during the last 3 weeks with hens fed on ulvan. The hen-day laying rate was significantly higher ($P < 0.05$) in the birds fed with 0.5, 0.8, and 1% ulvan at 66 and 68 weeks of age, and 0.1, 0.5, 0.8, and 1% ulvan also increased the laying rate at 67 weeks of age. We also calculated the total egg production (Fig. 2) and found that all groups added with ulvan (D2 to D6) were significantly higher than the control group (D1) ($P < 0.05$). Furthermore, egg production rates raised as the amount of ulvan additive increased among these groups. This clearly indicated the positive effect of ulvan on the laying rate. As shown in Table 3 and Fig. 3, the total egg weight and FCR were affected ($P < 0.05$) by dietary treatments. Egg weight was 1.41 kg (24 birds) for 1% ulvan treatment at age of 66 weeks which was much higher than the control group (1.25 kg), and 0.1 and 1% ulvan-added diets were increased at age of 68 weeks ($P < 0.05$). Similarly, during the whole experimental period (61 to 68 weeks) (Fig. 3b), egg weight tends to be enhanced with 0.1 and 1% of ulvan added in the daily diet ($P < 0.05$). The hens fed with 1% ulvan diet had the best feed efficiency compared to other five experimental groups at the age of 64 and 68 weeks as well as 61 to 68 weeks (Fig. 3d) ($P < 0.05$). However, throughout the experimental period (61 to 68 weeks), feed intake and average egg weight were not influenced by the dietary ulvan level ($P > 0.05$).

Effects of ulvan on egg quality of laying hens

The different egg quality parameters including egg-shaped index, eggshell strength, egg albumen height, Haugh unit, yolk color, and total cholesterol content of yolk are presented in Fig. 4. The results showed that ulvan can significantly affect eggshell strength (64 weeks), yolk color (64 and 68 weeks), and total cholesterol content of yolk (64 and 68 weeks). Compared to the control group (29.57 N), adding 0.8% (34 N) and 1% (34.41 N) ulvan into the daily diets can significantly enhance eggshell strength (Fig. 4b) in the last week of our experiment ($P < 0.05$). The yolk color (Fig. 4e) was also influenced by feeding with ulvan diets than control treatment ($P < 0.05$), and adding 0.5 to 1% ulvan can significantly deepen yolk color into red tendency. In addition, ulvan supplementation in hens' diets markedly decreased the total cholesterol content of yolk ($P < 0.05$) (Fig. 4f). Compared to the control group, except for 0.1% ulvan treatment at the middle of the experiment (64 weeks), all other experimental treatments showed favorable changes in the total cholesterol content of yolk ($P < 0.05$). However, egg-shaped index, egg albumen height, and Haugh unit showed no great variation depending on the ulvan ($P > 0.05$) (Fig. 4a, c, d).

Table 2 Effects of dietary ulvan levels on egg production of laying hens for 61–68 weeks of age (%). D1–D6 were applied at the following levels: 0 (D1), 0.05% (D2), 0.1% (D3), 0.5% (D4), 0.8% (D5), and 1% (D6) of the basic diets fed on laying hen. Means within the same row showing different lowercase letters are significantly different ($P < 0.05$). Values presented as mean ± SE

Item	Treatment						F value$^{(df)}$	P value
	D1	D2	D3	D4	D5	D6		
61 weeks	83.41 ± 0.46	84.49 ± 1.95	83.75 ± 1.74	83.05 ± 1.29	84.03 ± 1.18	83.52 ± 1.33	$0.413^{(5,30)}$	0.835
62 weeks	81.94 ± 1.49	82.9 ± 2.11	84.72 ± 2.56	84.38 ± 3.64	84.72 ± 2.32	85.76 ± 1.89	$0.599^{(5,30)}$	0.701
63 weeks	81.77 ± 1.29	81.32 ± 2.42	82.89 ± 3.79	81.94 ± 2.45	82.78 ± 2.78	84.03 ± 1.91	$0.991^{(5,30)}$	0.446
64 weeks	79.76 ± 2.14	82.48 ± 2.69	81.42 ± 2.99	83.17 ± 1.97	83.82 ± 1.99	83.25 ± 2.29	$0.742^{(5,30)}$	0.599
65 weeks	78.93 ± 1.69	81.06 ± 2.27	82.86 ± 3.71	82.64 ± 2.27	80.27 ± 1.76	84.09 ± 1.63	$0.460^{(5,30)}$	0.801
66 weeks	75.00 ± 3.31^{b}	80.03 ± 1.96^{ab}	80.09 ± 3.22^{ab}	83.56 ± 0.56^{a}	84.94 ± 1.79^{a}	85.85 ± 2.11^{a}	$2.997^{(5,30)}$	0.025
67 weeks	77.36 ± 0.49^{b}	80.92 ± 1.65^{ab}	82.19 ± 1.87^{a}	83.33 ± 1.32^{a}	83.33 ± 2.11^{a}	84.72 ± 1.39^{a}	$2.769^{(5,30)}$	0.046
68 weeks	75.89 ± 3.41^{b}	79.74 ± 1.48^{ab}	80.75 ± 1.67^{ab}	82.06 ± 2.09^{a}	82.82 ± 1.74^{a}	83.04 ± 1.12^{a}	$2.884^{(5,30)}$	0.037

Values followed by no letters or the same lowercase letters in the same row showed no significant difference. Values within the same row showing different lowercase letters are significantly different, where $P < 0.05$

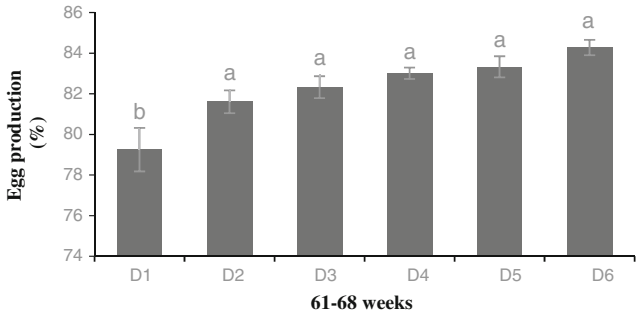

Fig. 2 Laying hens were fed with six different diets. The whole period (61–68 weeks) of the egg production was calculated. D1–D6 mean ulvan dosage of 0, 0.05, 0.1, 0.5, 0.8, and 1% of the laying hen groups. Different letters indicate statistical differences ($P < 0.05$) among the groups

Effects of ulvan on immunity function of laying hens

As shown in Table 4, we determined the content of IL-6, IFN-γ, IgG, and Flu-A-Ab in laying hens' serum. We analyzed the two-factor interaction of time and ulvan level at the age of 64 and 68 weeks. We found that IL-6 and IFN-γ were both affected significantly by the time × ulvan level ($P < 0.05$). The D6 group had the highest level of IL-6 at 68 weeks. Similarly, the D5 group had a higher level of IFN-γ than the other groups at 64 weeks. Moreover, the content of IFN-γ in serum at 64 weeks was markedly higher than 68 weeks ($P < 0.05$). The effect of ulvan diets on antibody production against influenza A virus (Flu-A-Ab) and immunoglobulin G (IgG) is also shown in Table 4, but ulvan diets and feeding time had no significant effect, and there is no reciprocal effect on the time × ulvan level.

Effects of ulvan on antioxidant capacity of laying hens

The effects of ulvan and time on total antioxidant capacity (T-AOC), MDA, and SOD in serum of laying hens are presented in Table 5. The results showed that there is a significant interaction ($P < 0.05$) on the time × ulvan level with T-AOC, SOD, and MDA. T-AOC activity of laying hens detected at 64 weeks was much lower than 68 weeks ($P < 0.01$), and T-AOC in all groups with different doses of ulvan were increased compared with the control group. Similar to T-AOC, the SOD activity of the laying hens under the control condition varied significantly from 64 to 68 weeks ($P < 0.01$). After feeding 8 weeks with ulvan diets, the SOD activity was much higher than feeding 4 weeks during the experiment. On the other hand, compared with the control group, other five ulvan groups had higher

Table 3 Effects of dietary ulvan levels on laying performance of laying hens for 61–68 weeks. D1–D6 were applied at the following levels: 0 (D1), 0.05% (D2), 0.1% (D3), 0.5% (D4), 0.8% (D5), and 1% (D6) of the basic diets. Means within the same row showing different lowercase letters are significantly different ($P < 0.05$). Values presented as mean ± SE

Item	Treatment						F value$^{(df)}$	P value
	D1	D2	D3	D4	D5	D6		
Average daily feed intake (g)								
62 weeks	109.38 ± 0.60	110.73 ± 0.36	110.32 ± 0.27	108.95 ± 0.23	109.89 ± 0.11	109.68 ± 0.78	$1.946^{(5,30)}$	0.116
64 weeks	110.57 ± 1.79	112.33 ± 1.10	109.91 ± 0.46	108.67 ± 0.14	109.76 ± 0.02	109.40 ± 0.67	$1.840^{(5,30)}$	0.135
66 weeks	111.42 ± 1.17	113.82 ± 3.12	119.20 ± 4.04	116.23 ± 0.74	112.81 ± 0.56	117.74 ± 1.43	$1.789^{(5,30)}$	0.145
68 weeks	109.91 ± 0.73	111.29 ± 1.17	111.38 ± 0.33	110.79 ± 0.64	109.47 ± 0.13	108.78 ± 1.08	$1.822^{(5,30)}$	0.139
Total egg weight (kg)								
62 weeks	1.24 ± 0.02	1.28 ± 0.05	1.23 ± 0.07	1.25 ± 0.04	1.19 ± 0.04	1.31 ± 0.03	$0.709^{(5,30)}$	0.621
64 weeks	1.18 ± 0.02	1.33 ± 0.03	1.18 ± 0.07	1.17 ± 0.05	1.20 ± 0.02	1.30 ± 0.03	$2.540^{(5,30)}$	0.050
66 weeks	1.25 ± 0.03^{b}	1.34 ± 0.02^{ab}	1.30 ± 0.05^{ab}	1.35 ± 0.03^{ab}	1.29 ± 0.04^{ab}	1.41 ± 0.03^{a}	$2.545^{(5,30)}$	0.049
68 weeks	1.23 ± 0.04^{b}	1.33 ± 0.02^{ab}	1.34 ± 0.05^{a}	1.27 ± 0.02^{ab}	1.24 ± 0.02^{b}	1.35 ± 0.02^{a}	$2.986^{(5,30)}$	0.039
Average egg weight (g)								
62 weeks	64.81 ± 0.20	64.20 ± 0.51	64.29 ± 0.39	64.66 ± 0.46	65.00 ± 0.44	64.45 ± 0.52	$0.492^{(5,30)}$	0.779
64 weeks	66.52 ± 1.12	65.51 ± 2.30	65.06 ± 0.40	66.98 ± 0.91	63.81 ± 0.83	62.34 ± 0.95	$1.952^{(5,30)}$	0.115
66 weeks	64.98 ± 0.69	64.26 ± 0.45	64.68 ± 0.46	64.17 ± 0.59	63.98 ± 0.43	63.87 ± 0.82	$0.507^{(5,30)}$	0.769
68 weeks	64.23 ± 0.39	63.64 ± 0.40	64.70 ± 0.22	64.17 ± 0.40	64.07 ± 0.46	63.48 ± 0.57	$1.064^{(5,30)}$	0.400
Feed conversion ratio (FCR) (gg^{-1})								
62 weeks	2.10 ± 0.04	2.08 ± 0.09	2.17 ± 0.12	2.08 ± 0.07	2.22 ± 0.08	2.01 ± 0.04	$0.855^{(5,30)}$	0.522
64 weeks	2.26 ± 0.04^{a}	2.15 ± 0.05^{ab}	2.24 ± 0.14^{a}	2.02 ± 0.10^{ab}	2.19 ± 0.04^{ab}	1.99 ± 0.04^{b}	$2.698^{(5,30)}$	0.044
66 weeks	2.13 ± 0.05	2.04 ± 0.06	2.11 ± 0.12	2.07 ± 0.04	2.09 ± 0.05	2.00 ± 0.03	$1.067^{(5,30)}$	0.398
68 weeks	2.13 ± 0.08^{a}	2.00 ± 0.03^{ab}	1.99 ± 0.07^{ab}	2.08 ± 0.03^{ab}	2.11 ± 0.04^{ab}	1.93 ± 0.05^{b}	$3.124^{(5,30)}$	0.029

Values followed by no letters or the same lowercase letters in the same row showed no significant difference. Values within the same row showing different lowercase letters are significantly different, where $P < 0.05$

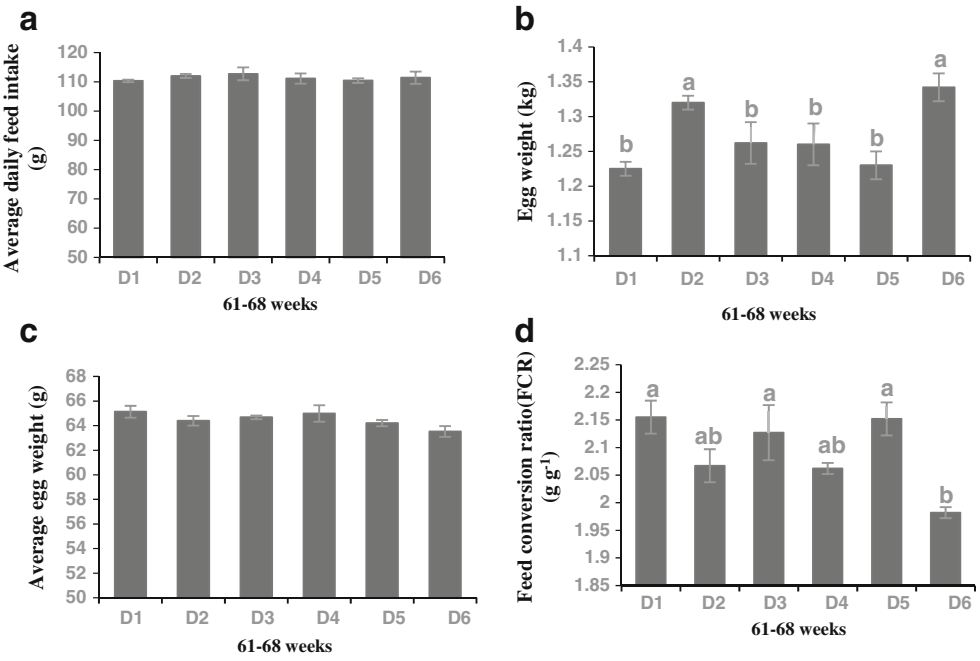

Fig. 3 Laying hens were fed with six different diets. Effects of ulvan on average daily feed intake (**a**), total egg weight (**b**), average egg weight (**c**), and feed conversion ratio (**d**) in the whole period (61–68 weeks) of the laying hens. D1–D6 mean ulvan adding dosage of 0, 0.05, 0.1, 0.5, 0.8, and 1%. Different letters indicate statistical differences ($P < 0.05$) among the groups

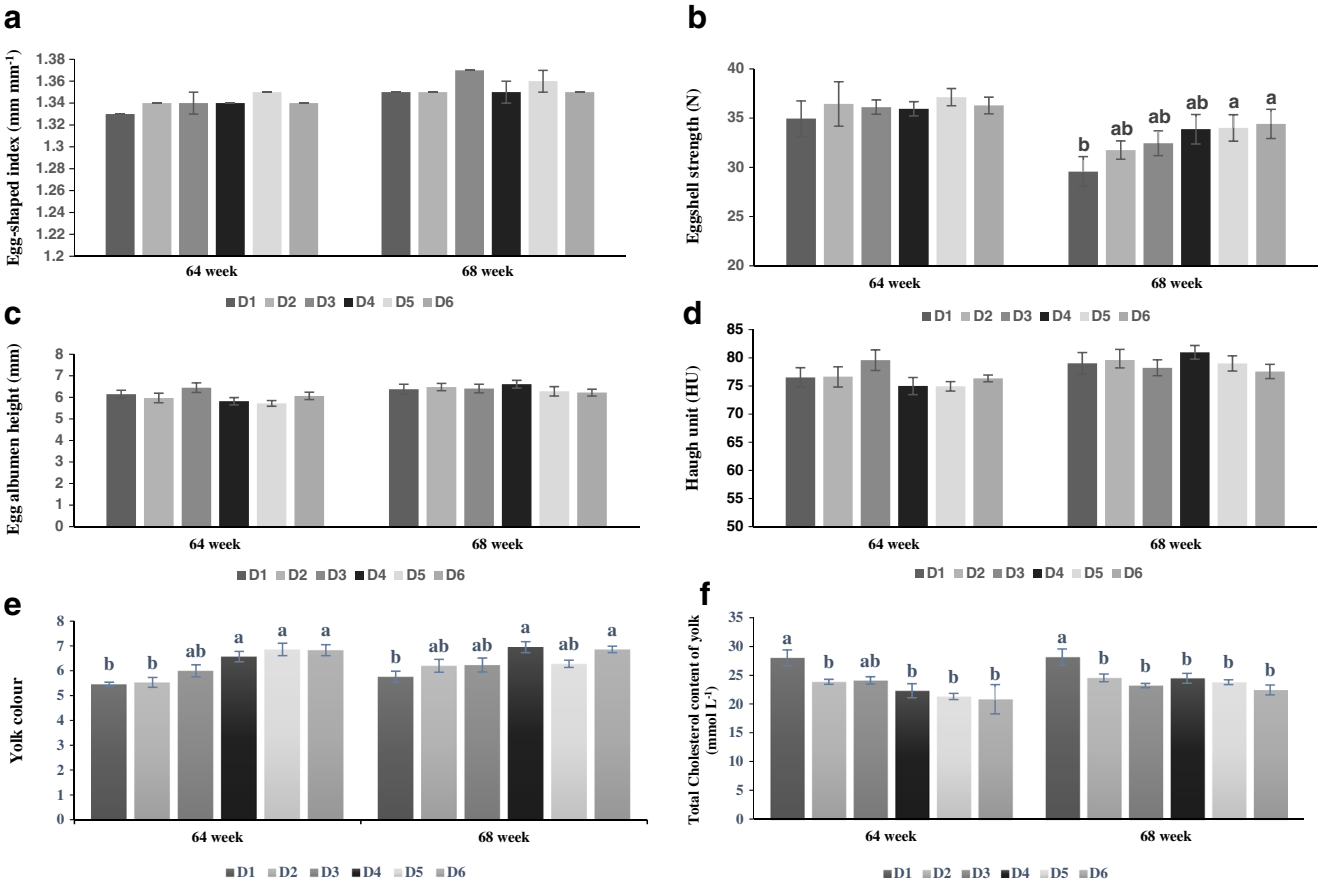

Fig. 4 Laying hens were fed with six different diets. Effects of ulvan on egg quality parameters at 64 and 68 weeks of laying hens, including egg-shaped index (**a**), eggshell breaking strength (**b**), albumen height (**c**), Haugh unit (**d**), egg yolk color (**e**), and egg cholesterol content (**f**). D1–D6 mean ulvan adding dosage of 0, 0.05, 0.1, 0.5, 0.8, and 1%. Different letters indicate statistical differences ($P < 0.05$) among the groups

Table 4 Effects of time and ulvan level on immunity function of laying hens. D1–D6 were applied at the following levels: 0 (D1), 0.05% (D2), 0.1% (D3), 0.5% (D4), 0.8% (D5), and 1% (D6) of the basic diets fed on laying hen

Items		IL-6 (ng L^{-1})	IFN-γ (pg mL^{-1})	IgG (μg mL^{-1})	Flu-A-Ab (pg mL^{-1})
64 weeks	D1	11.95	47.02	1.05	15.45
	D2	11.83	50.18	1.02	20.51
	D3	16.03	36.4	1.11	19.73
	D4	8.58	55.35	1.12	20.23
	D5	12.69	64.5	1.13	23.93
	D6	10.39	46.98	1.13	16.36
SEM		0.81	3.38	0.02	0.95
68 weeks	D1	12.85	29.41	1.1	14.69
	D2	10.93	41.67	1.05	14.36
	D3	14.41	36.21	1.1	18.41
	D4	11.19	42.18	1.1	17.21
	D5	14.32	52.71	1.18	19.24
	D6	17.02	44.4	1.08	23.76
SEM		0.61	2.93	0.02	1.03
Time	64 weeks	11.92	50.08	1.1	19.37
	68 weeks	13.13	41.1	1.11	17.95
SEM		0.68	3.05	0.02	0.96
Level	D1	12.41	44.61	1.08	15.08
	D2	11.39	45.93	1.04	17.44
	D3	15.23	36.31	1.11	19.07
	D4	9.89	42.39	1.12	18.73
	D5	12.51	58.61	1.16	21.59
	D6	13.71	45.7	1.11	20.06
SEM		1.17	5.28	0.03	1.66
df	Time	(1,59)	(1,59)	(1,59)	(1,59)
	Level	(5,59)	(5,59)	(5,59)	(5,59)
	Time × level	(6,59)	(6,59)	(6,59)	(6,59)
F value	Time	1.592	4.321	0.082	1.104
	Level	2.463	1.911	1.334	1.825
	Time × level	2.317	2.312	1.125	1.705
P value	Time	0.213	0.043	0.776	0.298
	Level	0.044	0.108	0.264	0.124
	Time × level	0.046	0.047	0.36	0.138

activity of SOD but not significantly ($P > 0.05$). Also, ulvan treatments changed the MDA activity of laying hens, and these changes were highly dependent on the level of ulvan ($P < 0.05$). The activity of MDA was significantly decreased at 68 weeks in ulvan diets of laying hens, especially in the D5 and D6 groups.

Discussion

Laying performance, including egg production rate, average daily feed intake, egg weight, average egg weight, and FCR, plays an important role in poultry farm. The data obtained in this study revealed that ulvan exhibited favorable effect on egg production, egg weight, and FCR. We speculated these results might be due to effects of ulvan on gut morphology and function of gastrointestinal digestion and absorption of laying hens (Bobin-Dubigeon et al. 1997). Other groups have found that ulvan cannot be digested in the gastrointestinal tract and therefore may be regarded as a good source of dietary fiber and a potential source of prebiotics (Bobin-Dubigeon et al. 1997; Lahaye et al. 1998; Fitzgerald et al. 2011). The small intestine is the primary organ for digestion and absorption, and normal function and structure of the intestinal mucosa are the basic guarantee for animals fully digesting and absorbing nutrients (Santin et al. 2001). In the current study, ulvan enhanced the function of small intestine and regulated the digestive system, which leads to the positive effect on egg production, egg weight, and FCR. This finding provides scope for adding ulvan in laying hens' daily diet had a good effect on the

small intestine, especially, the digestion function. However, feed intake and average egg weight were not influenced by the dietary ulvan level. Similar results were observed in other studies in which the feed was supplemented with red, brown, or green seaweeds such as *Macrocystis pyrifera* and *Sargassum sinicola* (Carrillo et al. 2008). Taken together, our results demonstrated that ulvan did not increase the intake, but the egg weight of hens was enhanced, which is of great benefit to farmers.

The results showed that adding ulvan into the daily diets can significantly enhance eggshell strength compared with that of the control group. Manangi et al. (2015) has reported that eggs from hens fed either no supplemental trace minerals or lower levels of supplemental inorganic trace minerals had lower shell thickness and seaweeds can be a source of minerals (Cabrita et al. 2016). So ulvan containing abundant sulfates makes a difference to shell thickness that leads to an enhanced eggshell strength. The yolk color was also influenced by feeding with ulvan diets. Several researchers have reported that macroalgae in poultry diets can be effectively used as a natural pigment source, both in the egg yolk and broiler skin (Sardi et al. 2006; Carrillo et al. 2008; Park et al. 2015).

Eggs are recognized as nutritious foods, but egg cholesterol is higher than most animal products. Many researchers have tried to decrease the amount of cholesterol in egg yolk due to its risk for cardiovascular disease in humans (Elkin 2006). Many marine resources have attracted attention in the search for antihyperlipidemic compounds to develop new drugs and health foods (Silva et al. 2013). In our study, except for 0.1% ulvan treatment at the middle of the experiment (64 weeks), ulvan supplementation markedly decreased the total cholesterol content of yolk (Fig. 4f). These results suggest that ulvan reduced the egg cholesterol content. Previous studies have reported that ulvan from *Ulva pertusa* was found to be a potential antihyperlipidemic agent and had significantly reduced serum triglyceride (TG) and total and low density lipoprotein cholesterol (LDL cholesterol) and elevated high density lipoprotein cholesterol (HDL cholesterol) in mice (Yu et al. 2003; Wijesekara et al. 2011). Qi and Sheng (2015) reported that high sulfate-content ulvan had the capacity to decompose cholesterol that may be through upregulation of the expression of ileum farnesoid X receptor (FXR) and hepatic cholesterol 7-alpha hydroxy-lase (CYP7A1) genes and downregulation of the expression of 1-BABP and hepatic FXR.

Many sulfated polysaccharides have been reported to have positive effects on the modulation of the immune system in vitro (Zhao et al. 2013; Wang et al. 2015a; Geng et al. 2016; del Rocío Quezada-Rodríguez and Fajer-Ávila 2017). IL-6 and IFN-γ are cytokines for the induction of cellular immunity (Yuan et al. 2015a). Flu-A-Ab and IgG were also investigated by testing ulvan's capacity to stimulate the humoral immune response. In our study, when we fed the laying hens for 8 weeks with the ulvan diets, we found that interleukin-6 (IL-6) and interferon-γ (IFN-γ) were significantly increased, especially at higher doses of ulvan. There were significant interactions between ulvan diets and feeding time for IL-6 and IFN-γ concentrations during 8 weeks, which indicated that the length of feeding time also has a certain effect on the content of IL-6 and IFN-γ. But the mechanism requires further study. Among previous studies, Cui et al. (2015) indicated that sulfated modification can improve the immunocompetence of purified *Lycium barbarum* polysaccharides by promoting lymphopoiesis and T lymphocyte differentiation as well as increasing IL-2, IL-6, IFN-γ, and TFN-α production in vitro. There was a strong relationship between the activities and high molecular weight. Sun et al. (2012) also reported that polysaccharides isolated from *Porphyridium cruentum* showed clear antitumor and immunomodulatory activities. Moreover, Shang et al. (2015) found that fructooligosaccharide (FOS) and *Salmonella enteritidis* LPS challenge established significant differences in the immune responses in broiler chickens.

It has been proved that the addition of sulfated polysaccharides extracted from *Ulva* (*Enteromorpha*) *prolifera* stimulated the production of macrophages and increased cytokine expression (Kim et al. 2011). Geng et al. (2016) also found that pine pollen sulfated polysaccharide (SPPM60-D) activates mouse macrophage through the signal pathway of SPPM60-D → TLR4 → PI3K → PLC → IP3 → IP3R → CRAC to increase intracellular free calcium concentration ($[Ca^{2+}]$) and further promote murine macrophages to secrete IL-1, IL-6, and TNF-α. Other studies have shown that sulfated polysaccharide extracts from marine algae might have a certain effect on the immune response genes (Bahar et al. 2012; Leiro et al. 2007). Nevertheless, ulvan cannot influence Flu-A-Ab and IgG during the experiment.

As attention has been focused on natural antioxidants in past years, several natural sulfated polysaccharides, including ulvan, were evaluated for their antioxidant activity. Qi et al. (2006) reported that different sulfate content ulvans showed different antioxidant activities; furthermore, high sulfate content ulvan showed stronger antioxidant activity than natural ulvan. Interestingly, ulvan from *U. pertusa*, as well as acetylated and benzoylated ulvans, were found to have antioxidant activity including scavenging activity against hydroxyl radicals, reducing power and chelating ability (Qi et al. 2005). Different doses of ulvan had a considerable impact on serum oxidative stability as measured by T-AOC, SOD, and MDA values. T-AOC can directly reflect the antioxidant enzyme activity and the status of antioxidant system, and its content was positively correlated with cellular antioxidant capacity. MDA is regarded as the main product of the endogenous lipid peroxidation and has been used as markers of oxidative stress. SOD is very important for eliminating free radicals. SOD activity indicates the ability of against oxidative stress reaction in cells. T-AOC, SOD, and MDA often cooperate with each other to test the oxidation state in vitro and in vivo. In our

Table 5 Effects of time and ulvan level on antioxidant capacity of laying hens. D1–D6 were applied at the following levels: 0 (D1), 0.05% (D2), 0.1% (D3), 0.5% (D4), 0.8% (D5), and 1% (D6) of the basic diets

Items		T-AOC (U mL^{-1})	MDA (U mL^{-1})	SOD (U mL^{-1})
64 weeks	D1	20.38	4.66	9.68
	D2	24.28	4.71	11.89
	D3	25.24	3.37	11.55
	D4	23.12	2.92	12.36
	D5	28.07	4.58	11.5
	D6	27.65	4.83	11.99
SEM		1.22	0.28	0.37
68 weeks	D1	28.12	6.38	16.29
	D2	33.57	4.1	19.2
	D3	34.46	5.85	14.72
	D4	34.48	5.46	18.86
	D5	32.7	2.72	16.56
	D6	34.82	1.73	18.81
SEM		1.48	0.45	0.73
Time	64 weeks	25.07	4.18	11.5
	68 weeks	33.58	4.38	17.41
SEM		1.46	0.35	0.57
Level	D1	24.25	5.26	12.99
	D2	30.59	4.41	15.55
	D3	31.52	4.88	13.14
	D4	27.97	2.33	15.62
	D5	30.39	3.65	14.04
	D6	31.24	5.15	15.41
SEM		2.47	0.6	0.98
df	Time	(1,71)	(1,71)	(1,71)
	Level	(5,71)	(5,71)	(5,71)
	Time × level	(6,71)	(6,71)	(6,71)
F value	Time	16.907	0.159	53.688
	Level	1.206	3.468	1.536
	Time × level	3.823	2.916	10.228
P value	Time	< 0.01	0.692	< 0.01
	Level	0.316	0.008	0.191
	Time × level	0.003	0.014	< 0.01

study, the feeding time and ulvan level interplayed the content of T-AOC, SOD, and MDA. T-AOC and SOD activity of laying hens detected at 64 weeks was much lower than 68 weeks, which may be due to a time accumulation effect. Shang et al. (2016) found that crude polysaccharides extracted from *Gynostemma pentaphyllum* (GPMPP) which was similarly composed of rhamnose, arabinose, xylose, mannose, glucose, and galactose significantly increased the T-AOC level and decreased the MDA level. Qi and Sheng (2015) found high sulfate-content derivative of polysaccharide (HU) from *U. pertusa* could increase significantly the levels of SOD. Overall, high sulfate content and the monosaccharide composition of ulvan result in the antioxidant function for laying hens.

In conclusion, ulvan at concentrations of 0.5, 0.8, and 1% can significantly improve the laying rates ($P < 0.05$). Higher concentration (1%) generally increased the egg weight and decreased the feed conversion ratio of hens ($P < 0.05$). 0.5 to 1% concentrations of ulvan also improved eggshell strength ($P < 0.05$) and lead yolk color to red tendency and significantly lower cholesterol level of yolk ($P < 0.05$). In addition, the level of IFN-γ and IL-6 in serum of 0.8 to 1% groups was also increased, and the concentration of T-AOC and SOD in 0.5 to 1% groups was significantly higher than that of the control group ($P < 0.05$), MDA was also significantly decreased ($P < 0.05$). There was an interaction between the dosage and the feeding time, and the effect increased with the increase of feeding time.

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Compliance with ethical standards

Ethical statement All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institute of Zoology, Chinese Academy of Sciences (approval number: IOZ20170024).

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