



Low Level of Dietary Organic Trace Elements Improve the Eggshell Strength, Trace Element Utilization, and Intestinal Function in Late-Phase Laying Hens

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This study was conducted to evaluate the effects of organic trace elements (Cu, Fe, Zn, and Mn) on performance, egg quality, trace elements utilization, and intestinal function in late-phase laying hens. A total of 1,080 laying hens (Hy-line brown, 65 weeks old) were randomly assigned to four treatments with six replications of 45 layers each. The basal diet was prepared without adding exogenous trace elements. The control group was fed with a basal diet supplemented with 600 mg/kg of inorganic trace elements. The three treatment groups were fed basal diets supplemented with 300, 450, and 600 mg/kg organic trace elements (OTE300, 450, and 600), respectively. The results showed that there was no significant difference in growth performance among all treatments. However, OTE450 significantly improved the eggshell strength of laying hens ($p < 0.05$), but had no significant effects on haugh unit, egg yolk weight, eggshell weight, and eggshell thickness, compared with other groups. Moreover, compared with the control group, OTE450 significantly increased the contents of copper, iron, and zinc in serum ($p < 0.05$). Meanwhile, all of the trace elements had a lower deposition in the feces in organic trace elements groups ($p < 0.05$). Histological analysis showed that the addition of organic trace elements could significantly improve the villus height and villus concealment ratio ($p < 0.05$). In addition, the messenger RNA (mRNA) and protein expressions of divalent metal transporter 1 (*DMT1*), zinc transporter 1 (*ZnT-1*), and ferroportin 1 (*FPN1*) were the highest in the OTE450 group. In conclusion, OTE450 could improve egg quality, intestinal function, and trace element utilization efficiency. Thus, this study provides a theoretical basis for the application of low levels of organic trace elements in laying hens.

Keywords: organic trace element, egg quality, utilization, intestinal function, laying hen

INTRODUCTION

Trace elements, as coenzyme factors of many metal enzymes, play important roles in digestion, absorption, metabolism, immune response, and biosynthesis (1, 2). To meet the requirement of animals, inorganic trace elements were commonly supplemented in diets. However, inorganic trace elements are easy to interact with other compounds to form insoluble substances, resulting in a high supplemental amount in feed following low absorption efficiency in the intestine and more excretions into the environment (3, 4). Organic trace elements are complexes or chelates formed by inorganic trace elements and organic complexes, such as proteins, amino acids, organic acids, small peptides, polysaccharides, and derivatives. Organic trace elements have higher bioavailability than inorganic trace elements because they can be absorbed through other transport routes, such as amino acid transport channels (5). The study reported that dietary supplementation of organic trace elements could improve egg quality and intestinal health and increase serum mineral element levels (6). In addition, compared with inorganic trace elements, a lower level of organic trace elements in feed can improve the eggshell quality, antioxidant capacity, and immune function and reduce the excretion of mineral elements in feces without impacting animal production performance (7–9). It has been reported that compared with traditional inorganic form, dietary supplementation of amino acid chelated trace elements, such as copper, iron, zinc, and manganese could significantly improve chicken growth performance, antioxidant capacity, and trace element absorption and decrease mineral excretion (9–13).

Low levels of organic compound trace elements improve the eggshell quality, antioxidant capacity, immune function, and mineral deposition of aged laying hens (14). In the late stage of laying, the strength and thickness of the eggshell and the ability of hens to transport and absorb trace elements are reduced (15). Most studies focus on the effects of single trace element supplementation on late-phase laying hens (16, 17).

However, few studies focus on the effects of organic trace elements on late-phase laying hens. Moreover, there are few reports on the study of duodenal trace element transport. The purpose of this study was to study the effects of organic trace elements on laying performance, egg quality, trace element deposition, intestinal morphology, and related trace element transporters, and to find the optimal amount of organic trace elements for late-laying hens' production performance.

MATERIALS AND METHODS

Experimental Design and Layers Management

The layers used in this experiment were cared for in accordance with the guidelines for the care and use of laboratory animals described by the Guide for the Care and Use of Laboratory Animals and approved by the Committee on the Ethics of Shandong Agricultural University (ID: S20180058).

A total of 1,080 Hy-line brown laying hens with the same genetic background and comparable body weight (1.55 ± 0.02 kg)

TABLE 1 | Dietary components and nutrient levels in the basal diet (as fed-basis).

Ingredients	Content (%)	Nutrient levels	Contents
Corn	61.00	Metabolisable energy (MJ/kg)	11.15
Soybean Meal	25.00	Crude Protein, %	15.53
Soybean Oil	1.00	Calcium, %	3.78
Limestone	8.00	Phosphorus, %	0.58
Premix ^a	5.00	Non-phytate Phosphorus, %	0.32
Total	100.00	Methionine, %	0.35

^aThe premix provided the following per kilogram of diet: sodium chloride 3.2 g, vitamin A 7,000 IU, vitamin D₃ 2,250 IU, vitamin E 20 IU, vitamin K₃ 1.13 mg, vitamin B₁ 1.68 mg, vitamin B₂ 6 mg, vitamin B₃ 45 mg, vitamin B₅ 10 mg, vitamin B₆ mg, vitamin B₉ 0.75 mg, biotin 0.11 mg, and vitamin B₁₂ 0.01 mg.

TABLE 2 | The forms and levels of trace elements in diets (mg/kg).

Element	Control	OTE300	OTE450	OTE600
Copper	7.80	3.90	5.85	7.80
Iron	84.00	42.00	63.00	84.00
Zinc	72.00	36.00	54.00	72.00
Manganese	72.00	36.00	54.00	72.00
Selenium	0.48	0.24	0.36	0.48
Iodine	0.48	0.24	0.36	0.48

aged 65 weeks were arranged in metal cages (40 cm × 45 cm × 45 cm, Shandong Renhe mechanical equipment Co., Ltd, Taian, China). The temperature of the chicken house was controlled between 20 and 25°C, and the chicken house was exposed to light for 16 h every day. The layers were randomly divided into four treatments, each treatment with six replicates and 45 layers per replicate for a 10-week feeding trial following a 7-day adaptation. Layers were allowed *ad libitum* access to feed and water. The health status and mortality of laying hens were recorded in time. Throughout the trial period, egg number and egg weight were recorded every day. Egg-laying rate, average daily feed intake, and feed egg ratio were calculated every week in replicates.

The experimental diets were formulated according to NRC (18) except that the form of trace element was provided as an experimental design. The composition and calculated nutrient levels in the basal diet are shown in **Table 1**. The control group was fed with a basal diet supplemented with 600 mg/kg of inorganic trace elements, and all other groups were fed with a basal diet supplemented with 300, 450, and 600 mg/kg organic trace elements (OTE300, 450, and 600) (Fujian ShenNa Bioengineering Co., Ltd., Fujian, China). The organic trace elements used in this study were coated with hydroxymethyl cellulose and starch. The levels of inorganic trace elements and organic trace elements in diets are shown in **Table 2**. Before the 10-week trial period, a 1-week adaptation was used to change the commercial diet into an experimental diet.

Sampling Collection

At week 10, two laying hens with similar body weight (BW) were selected in each repeated and fasted for 12 h. After

TABLE 3 | Primer sequences used for quantitative real-time PCR (qRT-PCR).

Target Genes	Primer sequence (5' to 3')	Product size	Accession no.
DMT1	F:CTCAGCCATCGCCATCAACCT R:CTCCAGCTTCGCAGACCATA	124	EF635922
ZnT-1	F:ATCTGCGAGTGCCTTCTTCTCT R:ATGAACACTGATGGTAGCCTGGA	84	AJ619980
FPN1	F:GGAGACTGGGTGGACAAGAA R:GATGCATTCTGAACAACCAAGGA	67	NM_001012913.1
β-actin	F:CGGTACCAATTACTGGTGTAGATG R:GCCTTCATTACATCTACTCTGG	134	NM_205518.1

blood collection from the brachial wing vein, the laying hens were humanly sacrificed. The serum was stored at -20°C until analysis.

At the end of the experiment, feces were collected for 3 consecutive days with a feces collection board. Feces collected every 24 h were stored in polyethylene bags. The collected feces were dried at 65°C for 48 h and then dried at 105°C . After crushing, it passes through a 1 mm screen, the feces were stored at -20°C .

The duodenum was isolated under sterile conditions after slaughter, and part of the duodenum was intercepted and stored at -80°C for gene and protein analysis. Moreover, approximately 3 cm of the middle duodenum was fixed in 4% (w/v) of paraformaldehyde for analyzing intestinal morphology and immunohistochemical analysis.

Egg Quality Analysis

At the end of the experiment, six eggs in each replicate with similar size and weight were randomly selected to measure egg weight, albumen height, and yolk height with a full-automatic eggshell strength tester (EFG0503; Robot Machine Co., Ltd, Tokyo, Japan) and a multifunctional egg quality analyzer (EMT5200; Robot Machine Co., Ltd, Tokyo, Japan). In addition, the eggshell thickness was measured with an automatic eggshell thickness meter (Echometer, Model 1061; Robot Machine Co., Ltd, Tokyo, Japan). Moreover, eggshell and yolk weights were recorded.

Serum and Feces Trace Elements Analysis

Copper, iron, zinc, and manganese in feces and serum were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES, Optima 8300, Perkin Elmer, Waltham, MA, USA). Briefly, the sample was ashed and carbonized, dissolved with a 6 mol/L hydrochloric acid solution, and fixed with water into a 50 ml volumetric flask to be measured.

Duodenum Morphological Observation

The fixed duodenum was embedded in a fully automatic rotary paraffin slicer (HM355S, Burton International Trading Co., Ltd, China), sliced to a $5\ \mu\text{m}$ thickness using a microtome device. After that, the samples were dyed by an automatic dyeing machine (A81500101, Thermo Scientific, UK) and sealed with neutral resin. The morphology of the duodenum was observed

TABLE 4 | Effects of dietary organic trace elements supplementation on production performance of late-phase laying hens.

Items	Control	OTE300	OTE450	OTE600	SEM	P-value
Average laying rate, %	83.63	83.60	84.27	82.72	0.229	0.118
Average egg weight, g	59.50	60.75	60.08	60.92	0.239	0.130
Average daily feed intake, g	138.64	138.66	139.46	138.78	0.143	0.136
Feed/egg ratio	2.34	2.28	2.28	2.28	0.010	0.163

and photographed by microscope (Nikon elipse 80i, Japan). For each sample, 10 well-oriented villi and corresponding crypts were measured by an image analyzer (Lucia software, zdrahau), and the ratio of villi to crypts was calculated.

Immunohistochemical Analysis

Duodenal sections were prepared according to the standard immunohistochemistry (IHC) protocols. After the sections were dewaxed, the antigen was repaired by microwave oven in sodium citrate buffer (0.01 mol/L, pH = 6) for 5 min each time. The sections were repeated three times, then treated with 10% hydrogen peroxide for 1.5 h, and then incubated in 10% goat serum (ZSGB-BIO, Beijing, China) for 1 h to block nonspecific binding. Immunohistochemical analysis was performed using a kit (Polink-2) plus[®] Polymer HRP Detection system for rabbit primary antibody, PV-9001 (ZSGB-BIO, Beijing, China). After being washed with hydrochloric acid buffer, the above-prepared sections were incubated with antibodies Anti-DMT1 (1:100, bs-3577R, BIOSS, Beijing, China), Anti-ZnT-1 (1:100, bs-6440R, BIOSS, Beijing, China), and Anti-SLC40A1/FPN1 (1:100, 26601-1-AP, Proteintech, Wuhan, China) at 4°C overnight. Then, the slices were washed with phosphate-buffered saline (PBS), incubated with primary antibodies at 37°C for 1 h, and then incubated with Polink-2 plus Polymer HRP anti-rabbit secondary antibodies at 37°C for 1 h. After incubation, the slices were washed with PBS, soaked in diaminobenzidine (DAB) with a kit (DAB kit, TIANGEN PA110, Beijing, China) for 2 min, tested for immunostaining, and finally sealed with neutral resin. The immunoreactive substances were located with a bright field of vision using a microscope. Three stained sections of each sample were randomly selected for observation and shooting, and the images were analyzed by image analysis software (imagepro Plus6.0, media control, Yinquan, MD, USA). The total cross-sectional integrated optic density (IOD) was obtained and the staining intensities of divalent metal transporter 1 (DMT1), zinc transporter 1 (ZnT-1), and ferroportin 1 (FPN1) between the control group and the coating treatment were compared. The IOD of each sample is the average of the three stained sections.

Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was extracted from the duodenum of laying hens using Trizol total RNA extraction reagent according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using a reverse transcription Kit (PrimeScript™ RT Master Mix, RR036A, Applied, TaKaRa, Dalian, China). The quantitative real-time PCR (qRT-PCR) reaction was carried out with a quantitative real-time PCR master mix (RR390A, TaKaRa Bio Inc., Japan) in an ABI7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) in triplicate and repeated at least three times. β -Actin was used as an internal control. The relative expression of messenger RNA (mRNA) was calculated by the $2^{-\Delta\Delta CT}$ method. The primer sequence and product size are shown in Table 3.

Western Blot Analysis

Duodenal protein was extracted according to the manufacturer's instructions for lysate (Beyotime, Shanghai, China). The Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China) was used to determine the concentration, and the protein content of each sample was adjusted to 50 μ g. The proteins were separated by polyacrylamide gel electrophoresis and transferred to the immobilized protein transfer membrane (Solarbio, Beijing, China). After being washed three times with Western washing solution for 10 min each time and blocked using Protein Free Rapid Blocking Buffer (EpiZyme, Shanghai, China) for 20 min, the membranes were incubated with primary antibody: Anti-DMT1 (1:500, bs-3577R, BIOSS, Beijing, China), Anti-ZnT-1 (1:500, bs-6440R, BIOSS, Beijing, China), and Anti-SLC40A1/FPN1 (1:500, 26601-1-AP, Proteintech, Wuhan, China) overnight. Membranes were incubated with Anti-Rabbit IgG (1:2500, Beyotime, Shanghai, China) diluted with secondary antibody diluent (Beyotime, Shanghai, China) at 37°C for 2 h. Then, they were soaked in high-sensitivity luminescent reagent (BeyoECL Plus, Beyotime, Shanghai, China) and quantified by image software (Image Pro Plus 6.0, Media Cybernetics, Silver Spring, MD, USA). All western blot experiments were conducted with three biological repeats and repeated three times. All results were normalized to β -actin and the data were expressed as the relative values to those of the control group.

Statistical Analysis

All data were analyzed by using the general linear model (GLM) in SAS 9.4 (SAS Institute Inc., Cary, NC, USA), and differences among treatments were compared with Tukey's multiple range tests. Results were presented as the mean and standard error of the mean (SEM). All statements of significance are based on a probability of $p < 0.05$.

RESULTS

Production Performance

The production performance of laying hens is shown in Table 4. There was no significant difference in average egg production rate, average egg weight, average daily feed intake, and feed/egg ratio between all groups.

TABLE 5 | Effects of dietary organic trace elements supplementation on egg quality in late-phase laying hens.

Items	Control	OTE300	OTE450	OTE600	SEM	P-value
Eggshell breaking strength, N	39.34 ^b	38.32 ^b	41.76 ^a	37.75 ^b	0.468	0.005
Haugh unit	65.55	73.38	68.15	62.33	2.042	0.281
Egg yolk weight, g	18.11	17.63	17.59	17.35	0.169	0.475
Eggshell weight, g	6.45	6.31	6.15	6.22	0.068	0.453
Eggshell thickness, mm	0.30	0.29	0.32	0.30	0.005	0.332

^{a,b}Means without a common letter differed, $P < 0.05$.

TABLE 6 | Effects of dietary organic trace elements supplementation on serum trace elements in late-phase laying hens (μ g/ml).

Items	Control	OTE300	OTE450	OTE600	SEM	P-value
Copper	1.53 ^b	1.34 ^c	1.64 ^a	1.52 ^b	0.026	<0.001
Iron	9.40 ^b	8.45 ^c	10.41 ^a	9.65 ^b	0.152	<0.001
Zinc	7.48 ^b	6.52 ^c	8.61 ^a	7.05 ^b	0.177	<0.001
Manganese	0.63 ^{ab}	0.55 ^b	0.69 ^a	0.65 ^{ab}	0.019	0.039

^{a-c}Means without a common letter differed, $P < 0.05$.

TABLE 7 | Effects of organic trace elements supplementation on feces trace elements in late-phase laying hens (mg/kg).

Items	Control	OTE300	OTE450	OTE600	SEM	P-value
Copper	35.80 ^a	24.48 ^d	30.85 ^c	33.83 ^b	0.903	<0.001
Iron	1253.57 ^a	967.29 ^d	1003.91 ^c	1063.98 ^b	23.454	<0.001
Zinc	451.65 ^a	266.52 ^d	365.77 ^c	388.55 ^b	13.776	<0.001
Manganese	123.81 ^a	89.17 ^d	100.90 ^c	115.90 ^b	2.806	<0.001

^{a-d}Means without a common letter differed, $P < 0.05$.

Egg Quality Analysis

The egg quality of laying hens is shown in Table 5. Compared with the control group, the eggshell strength in the OTE450 group increased dramatically ($p < 0.05$), while OTE300 and OTE600 did not affect eggshell strength. Nevertheless, dietary organic trace elements supplementation had no obvious effects on haugh unit, yolk weight, eggshell weight, and eggshell thickness compared with those in the control group.

Serum and Feces Trace Element Analysis

As shown in Table 6, the serum copper concentration of the OTE450 group was the highest, while the content of the OTE300 group was the lowest ($p < 0.05$). There was no significant difference in serum copper concentration between the control and OTE600 groups. Moreover, iron and zinc concentrations have a similar trend with copper. However, the serum manganese concentration in the OTE450 group was significantly higher than that in the OTE300 group, but not significantly different from the OTE600 group and control group ($p < 0.05$). Trace elements

deposition in the feces of laying hens is shown in **Table 7**. All of the trace elements (copper, iron, zinc, and manganese) had a lower deposition in the feces in organic trace element groups, compared with the control group ($p < 0.05$). With the increase in the amount of organic trace elements, the content of copper, iron, zinc, and manganese in feces increased significantly ($p < 0.05$).

Duodenal Morphology

The effects of organic trace elements supplementation on the duodenal morphology of laying hens are shown in **Table 8** and **Figure 1**. The supplementation of organic trace elements can significantly improve the villus height and villus concealment ratio ($p < 0.05$), among which the OTE450 group is the highest, the OTE600 group is the second, and there is no significant difference between the control group and OTE300 group.

Expression of Trace Element Transporter

The immunohistochemical results of *DMT1*, *ZnT-1*, and *FPN1* in the duodenum are shown in **Table 9** and **Figures 2–4**. The supplementation of organic trace elements increased the expression of *DMT1*, *ZnT-1*, and *FPN1* in villi and crypts ($p < 0.05$).

The expression of *DMT1* in intestinal epithelial cells and crypts from high to low was OTE450 > OTE300 > control > OTE600 ($p < 0.05$). The expression of *ZnT-1* and *FPN1* in the control group was the lowest, followed by the OTE300 group, OTE450 group, and OTE600 group, but there was no significant difference between the three groups. The mRNA expressions of *DMT1*, *ZnT-1*, and *FPN1* in the layer duodenum are shown in **Figure 5**. The mRNA expression of *DMT1* in the OTE450 group was significantly higher than that in other groups ($p < 0.05$), and that in the OTE600 group was the lowest. The mRNA expression of *ZnT-1* in OTE450 was the highest, followed by that in OTE600 ($p < 0.05$), and then that in OTE300. The expression of *FPN1* was the highest in the OTE450 group ($p < 0.05$), followed by that in the OTE600 group, which was significantly higher than that in the control and OTE300 groups ($p < 0.05$). The protein expression of *DMT1*, *ZnT-1*, and *FPN1* in layer duodenum is shown in **Figure 6**. The expressions of *DMT1* and *ZnT1* were higher in the OTE450 group than that in other groups ($p < 0.05$), but there was no significant difference between the OTE300 group and the control group, which were significantly higher than that in the OTE600 group. The expression relationship of *FPN1* protein was OTE450 > OTE600 > OTE300 > control.

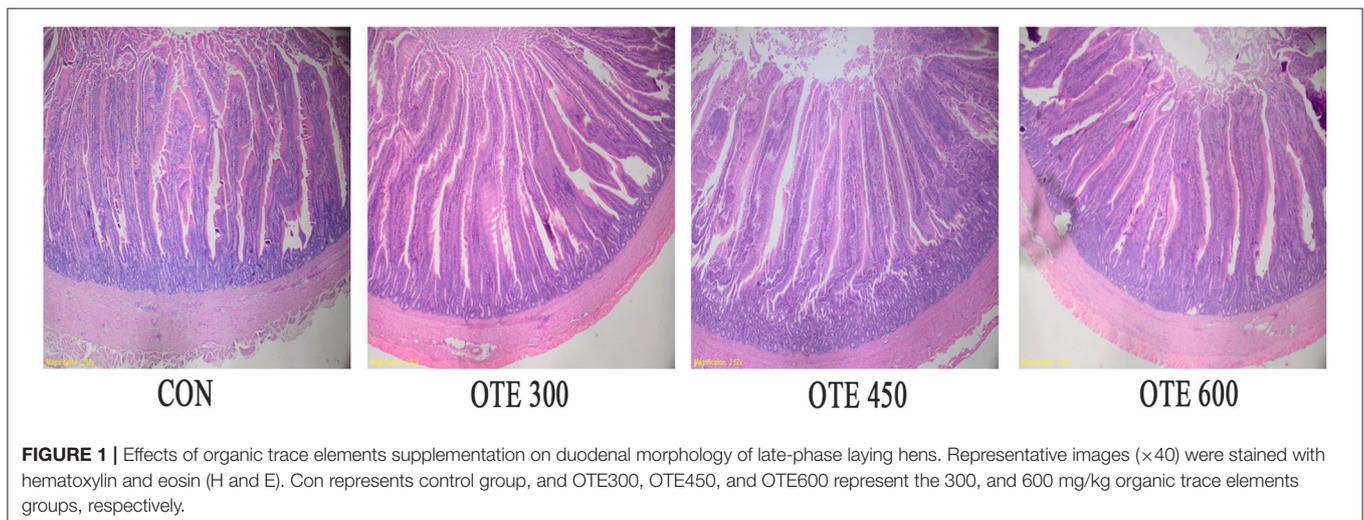
TABLE 8 | Effects of organic trace elements supplementation on duodenal morphology of late-phase laying hens.

Items	Control	OTE300	OTE450	OTE600	SEM	P-value
Villus height, μm	1237.3 ^c	1208.3 ^c	1419.9 ^a	1335.9 ^b	26.785	<0.001
Crypt depth, μm	204.8	207.6	190.2	202.5	2.664	0.073
Velvet concealed ratio	6.0 ^c	5.8 ^c	7.5 ^a	6.6 ^b	0.203	<0.001

^{a–c}Means without a common letter differed, $P < 0.05$.

DISCUSSION

Copper, iron, zinc, and manganese are essential trace elements for the production performance, reproduction, and metabolism of laying hens. They play important roles in laying, tissue mineral element precipitation, and metabolism (19, 20). In a 57-week-old Beijing Red Layer hens experiment, replacing 0–50% inorganic trace elements with organic compound trace elements did not affect the growth performance of layers (9). In addition, the study showed that 50% NRC (18) level of organic mineral elements supplementation to 26 weeks Roman layer diets did not influence feed intake, egg-laying rate, and feed egg ratio (21). Similarly, some studies also found that organic trace elements supplementation had no significant effect



on production performance compared with inorganic trace elements (22–24), which is consistent with the present study.

TABLE 9 | Effects of organic trace elements supplementation on cumulative optical density of divalent metal transporter 1 (DMT1), zinc transporter 1 (ZnT-1), and ferroportin 1 (FPN1) positive reactions in duodenum of late-phase laying hens ($\times 10^3$).

Items	Control	OTE300	OTE450	OTE600	SEM	P-value
DMT1						
Duodenal villi	79.1 ^c	94.3 ^b	139.1 ^a	39.5 ^d	10.801	<0.001
Duodenal gland	72.2 ^c	84.4 ^b	138.5 ^a	34.6 ^d	11.239	< 0.001
ZnT-1						
Duodenal villi	88.7 ^c	117.6 ^b	145.6 ^a	137.1 ^a	6.181	< 0.001
Duodenal gland	62.2 ^c	75.4 ^b	126.5 ^a	127.6 ^a	8.999	< 0.001
FPN1						
Duodenal villi	88.5 ^c	116.9 ^b	147.4 ^a	134.1 ^a	6.511	< 0.001
Duodenal gland	67.9 ^c	83.3 ^b	115.8 ^a	112.6 ^a	6.146	< 0.001

However, there are some inconsistent reports with this study. The supplementation of 80 mg/kg zinc-methionine in 49-week-old layer diets significantly decreased the feed intake and increased egg production (25). In addition, the supplementation of organic trace elements could increase the average daily feed intake and egg production of 30-week-old layers under high temperatures (26). These inconsistencies may be caused by the breed and age of laying hens, and the form and level of trace elements in their diets.

Copper, iron, zinc, and manganese play important roles in the formation of eggs. Copper can catalyze the formation of eggshell membrane fiber structure through lysyl oxidase, and then increase the toughness of the eggshell membrane (27). Iron mainly affects eggshell color by regulating the formation and transport of protoporphyrin IX (28, 29). Zinc is an important part of bicarbonate, which can affect the formation of calcium carbonate in the shell gland by providing carbonate ions, and finally participate in the formation of eggshell (19). Manganese affects the activity of 1, 3-glucuronosyltransferase (GLcAT-I) by regulating galactose, thereby changing the quality of eggshells (30).

It has been reported that organic trace element supplementation can improve the eggshell strength of eggs (31, 32). In addition, organic trace elements were supplemented

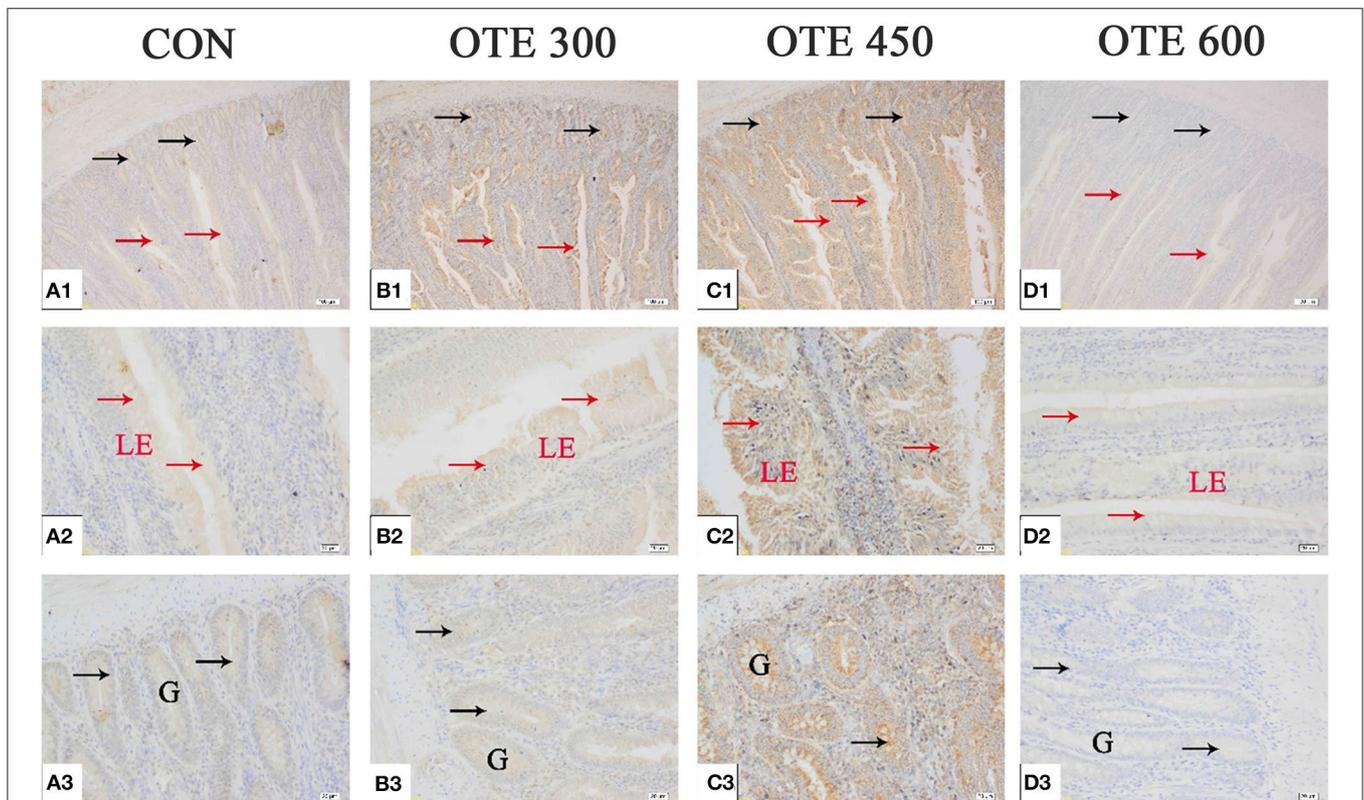
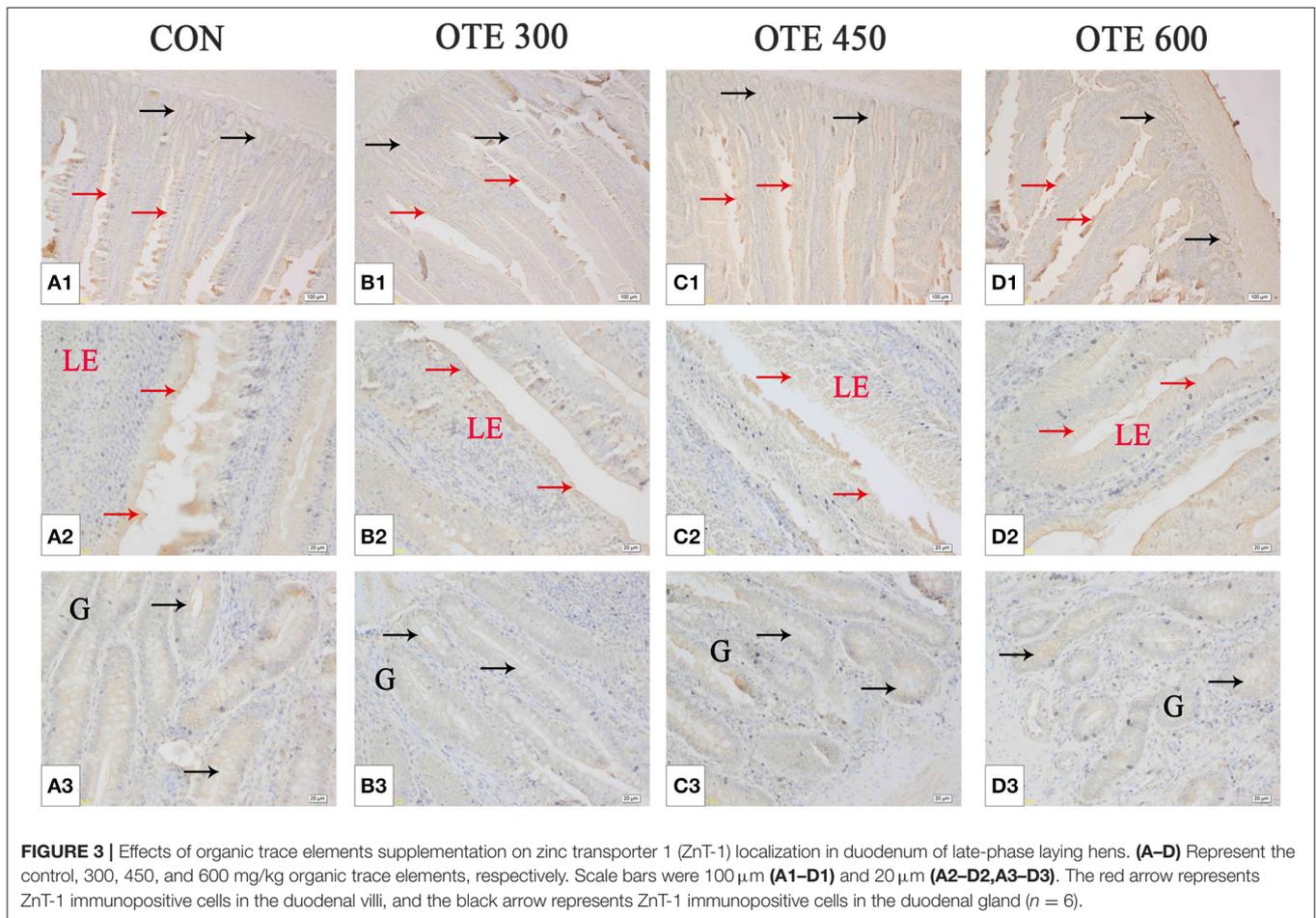


FIGURE 2 | Effects of organic trace elements supplementation on divalent metal transporter (DMT1) localization in duodenum of late-phase laying hens. (A–D) Represent the control, 300, 450, and 600 mg/kg organic trace elements groups, respectively. Scale bars were 100 μ m (A1–D1) and 20 μ m (A2–D2, A3–D3). The red arrow represents DMT1 immunopositive cells in the duodenal villi, and the black arrow represents DMT1 immunopositive cells in the duodenal gland ($n = 6$).

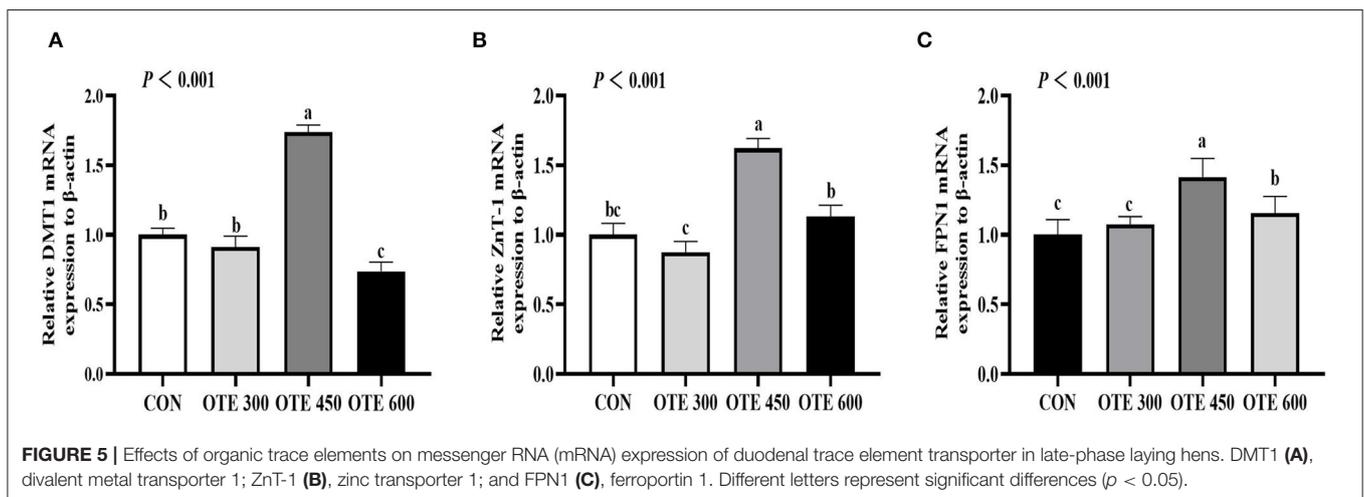
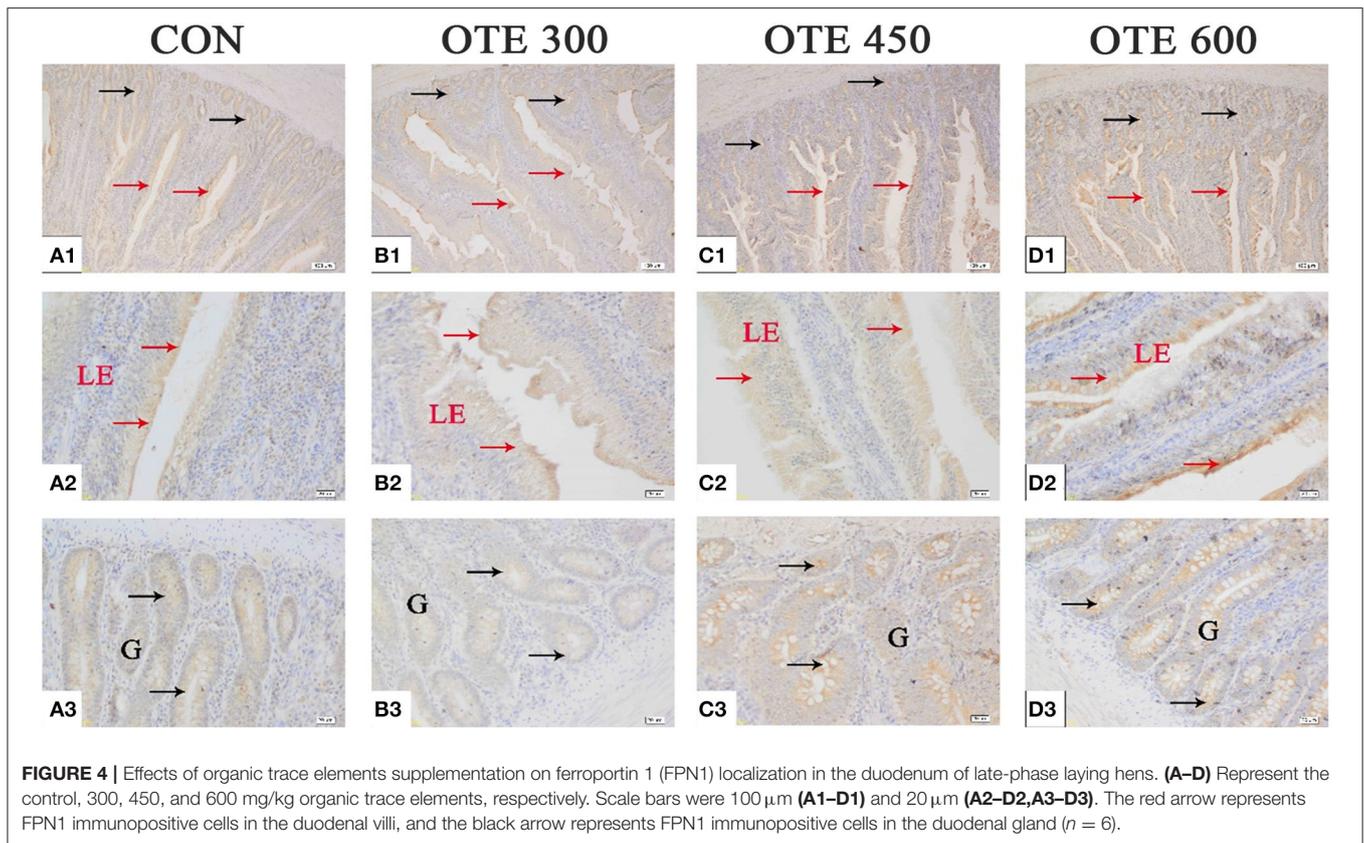


to a 47–62-week-old Hailan brown layer, which significantly improved the breaking strength and thickness of eggshells (33, 34). However, some studies reported that there was no significant difference in egg quality between organic trace elements and the inorganic trace elements group (8, 35). In this experiment, OTE450 supplementation significantly improved the strength of the eggshell but did not change the haugh unit, yolk weight, eggshell weight, and eggshell thickness. This is consistent with the study that the supplementation of organic trace elements promoted the fusion of the initial stage of the eggshell and improved the mechanical strength of the eggshell regardless of the thickness (32).

Organic trace elements have better bioavailability than inorganic trace elements. The supplementation of organic mineral elements has been proven to promote the absorption of trace elements and increase their levels in serum (36, 37). The supplementation of organic trace elements could significantly increase the serum contents of copper, iron, zinc, and manganese of 40-week-old broilers (38). Our results indicated that the content of serum trace elements was significantly increased by supplementing 450 mg/kg of organic trace elements. However, the contents of serum copper, iron, zinc, and manganese decreased in the OTE600 group. The reason

for this phenomenon may be that the 450 mg/kg organic trace elements supplementation is the optimal level for maintaining the homeostasis of serum trace elements. More and less organic trace element supplementation could break this homeostasis, which results in the decrease of serum trace element content. Compared with inorganic trace elements, adding a lower level of organic trace elements can reduce the excretion of mineral elements in feces without having adverse effects on poultry health and production (39). Similar to our results, organic trace elements significantly reduced the contents of copper, iron, zinc, and manganese in feces, which increased linearly with the increase of organic trace elements level. Above all, organic trace elements can reduce the excretion of trace elements in feces by improving the absorption of mineral elements in serum, and the OTE450 group showed optimal effects.

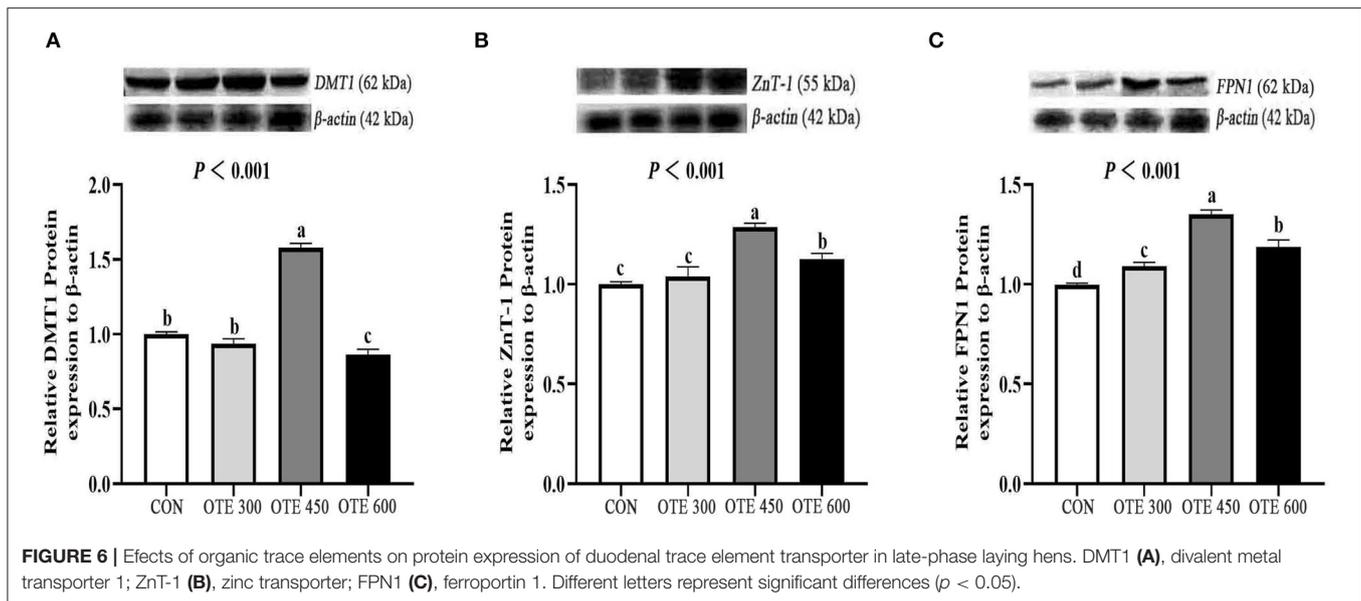
Minerals were mainly absorbed by the duodenum and then transported to the portal vein circulation (40). Villus height and crypt depth can be used to evaluate intestinal development, digestion, and absorption capacity (41). The villus height and crypt depth represent the digestive and absorptive capacity of the small intestine and the maturity of epithelial cells, while the villi to crypt ratio represents the digestive and absorptive function of the small intestine (42). The supplementation of glycine chelated



zinc can significantly improve the villus height and the ratio of the villus to the crypt in broilers (43). Our experiments showed that OTE450 and OTE600 significantly increased the villus height and the ratio of villus height to crypt depth compared with the control group. However, the villus height and ratio of villus height to crypt depth in the OTE600 group were lower than those in the OTE450 group. The phenomenon may be explained that compared with the OTE600 group, the OTE450 group has a better absorption capacity of trace elements. The study has

shown that the increase in villus length is related to the digestion and absorption of nutrients (44, 45). Therefore, the villus height and ratio of villus height to crypt depth were higher in the OTE450 group.

Trace element transporters include *DMT1*, *ZnT-1*, and *FPN1*. *DMT1* can use a proton gradient to drive the absorption of divalent metal ions, such as copper, iron, and zinc (46). *DMT1* was found in the apical membrane of intestinal cells and slightly expressed in crypts (47). Trace elements could be transported



by *DMT1* to small intestinal epithelial cells through microvilli (48, 49). *FPN1* is the only iron transporter in the intestinal tract in animals, which is located in the basal layer of duodenal intestinal cells to transport iron and manganese to blood (50–53). Similarly, as a zinc transporter, *ZnT-1* is widely expressed in the duodenal basement membrane and functions to transport zinc from intestinal epithelial cells to the blood. It was found that the mRNA expression of *DMT1* in broilers fed with organic manganese was significantly increased (54). Additionally, the supplementation of organic manganese significantly increased the mRNA and protein expression of *DMT1* and *FPN1* in 15-day-old broilers (55). Moreover, a report found that the mRNA expression of *FPN1* was significantly increased in 10-day-old layers fed with manganese (56). In addition, studies showed that zinc supplementation could increase the mRNA expression of *ZnT-1* in chicken, Caco-2 cells, and rat small intestine (57–59). In this study, the mRNA and protein expression of duodenal *DMT1*, *ZnT-1*, and *FPN1* increased in the organic trace element group, indicating that a low level of organic trace elements could promote the absorption and excretion of divalent metal ions in intestinal epithelial cells. In addition, the IOD value of IHC showed that the supplementation of organic trace elements significantly increased the expression of *DMT1*, *ZnT-1*, and *FPN1* in the duodenum. This is consistent with the results of western blot analysis.

CONCLUSIONS

In conclusion, the addition of 450 mg/kg of organic trace elements to the diet significantly improved the eggshell strength and serum trace element deposition of laying hens without affecting the production performance, increased the height of duodenal villi and the ratio of villi height to crypt depth, as well as the mRNA and protein expression of *DMT1*, *ZnT-1*, and

FPN1. This indicates that low levels of organic trace elements can improve eggshell strength, trace element utilization, and duodenal function.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on the Ethics of Shandong Agricultural University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

W-rY and S-zJ received funding. NJ and YL conceptualized and designed the study. XC and FZ conducted animal experiments, chemical analyses, and analyzed the data. XC wrote the original. NJ, S-zJ, and L-bH reviewed and revised the draft. All authors agreed to publish the manuscript.

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