



Original Research Article

Uterine inflammation status modulates eggshell mineralization via calcium transport and matrix protein synthesis in laying hens

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ABSTRACT

This study explored the effects of uterine inflammation on eggshell mineralization, ultrastructure and mechanical properties in laying hens modified by a lipopolysaccharide (LPS) challenge or dietary essential oil (EO) addition. In trial 1, a total of 72 Hy-line Brown layers at 36 wk of age were randomly assigned to 3 treatment groups ($n = 8$), where they were intravenously injected with phosphate buffered saline, LPS at 1 mg/kg body weight, or LPS 3 times at 24-h intervals. In trial 2, a total of 288 Hy-line Brown layers at 60 wk of age were randomly divided into 4 groups ($n = 8$), where they were fed basal diets supplemented with EO at 0, 50, 100 and 200 mg/kg for 12 wk. A uterine inflammation model was constructed with LPS treatment, indicated by the elevated expression of *IL-1 β* and *TNF- α* ($P < 0.05$) and lymphocyte infiltration. Uterine inflammation caused remarkable decreases in eggshell thickness and mechanical properties with structure deteriorations ($P < 0.05$). Uterine inflammation stimulated the expression of matrix proteins ovotransferrin (*TF*) and ovalbumin (*OVAL*), while depressing the mRNA levels of calbindin-1 (*CALB1*) and osteopontin in uterine mucosa ($P < 0.05$). In contrast, EO addition alleviated uterine inflammation, evidenced by depressed levels of *IL-1 β* and *IL-6* ($P < 0.05$). There was a significant elevation in shell thickness and breaking strength following EO intervention ($P < 0.05$), and these effects were maximized at addition of 100 mg/kg. Further, EO improved shell ultrastructure including more early fusion, less type B mammillae, and increased effective thickness ($P < 0.05$). The alleviated inflammation decreased the expression of *OVAL* and *TF*, whereas ion transport genes like *CALB1* and solute carrier family 26 member 9 were upregulated ($P < 0.05$). Our findings suggest that inflammatory status can impact uterine functions in calcium transport and the synthesis of matrix proteins especially such as *OVAL* and *TF*, which in turn modulates calcium precipitation and ultrastructure formation, thereby determining eggshell mechanical properties. These findings provide a novel insight into the uterine inflammation-mediated modifications of eggshell quality.

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1. Introduction

Cracked and broken eggs in the late phase can account for 12% to 20% of laying hen production (Travel et al., 2011), which limits extension of the laying cycle of commercial flocks and results in huge economic losses (Fathi et al., 2019; Gu et al., 2021). In practice, eggshell breakage mainly stems from infectious diseases and aging, which could be attributed to uterine inflammation and dysfunction (Feng et al., 2020; Jiang et al., 2021; Nii et al., 2014). However, the underlying mechanisms by which uterine inflammatory status mediate declines in the mechanical properties of eggshell remain to

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be explored. During eggshell biomineralization, calcium and bicarbonate ions provided through ionic transport and organic matrix are secreted into uterine fluid, where their precise interactions largely determine shell ultrastructure and strength (Brionne et al., 2014; Gautron et al., 2021; Yang et al., 2020). Shell ultrastructure, including membranes (inner and outer), mammillary layer, palisade layer, vertical crystal layer and cuticle, have been recognized as a major determinant of its mechanical properties and quality (Athanasidou et al., 2018). Thus, we hypothesize that uterine inflammatory status-induced adverse effects on the formation of eggshell structure are mediated mainly through interference with ionic transport and matrix protein synthesis, which subsequently alters mechanical properties and causes shell breakage.

Immune response and mucosal inflammatory status can be regulated by pro-inflammatory cytokines, whose sequential expression (*IL-1 β* , *IL-6*, *TNFSF15* and *IFN- γ*) in the uterus during different stages of eggshell formation (Elhamouly et al., 2018) indicates their potential roles in biomineralization. In contrast, pathogen-induced inflammation or *IL-1 β* treatment disturbs shell calcification, indicated by disrupted expression of ionic transporters (calbindin 1 [CALB1]) and matrix proteins (collagen type I) (Fan et al., 2014; Li et al., 2017; Nii et al., 2018; Qi et al., 2016). Between laying hens producing shells differing in breaking strength and ultrastructure, uterine transcriptomic analysis showed that differentially expressed genes were mainly annotated to the Gene Ontology (GO) terms or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways related to inflammation, such as regulation of *IL-1* production, arachidonic acid metabolism, and the mitogen-activated protein kinase signaling pathway (Zhang et al., 2019). Similarly, our previous study showed that age-related immune hypofunction and its resultant inflammatory state characterized by higher levels of pro-inflammatory cytokines may be associated with ultrastructure deteriorations and compromised mechanical properties of eggshell in the late phase of production (Feng et al., 2020). Of note, these inflammation-mediated structural variations were mainly reflected in the mammillary layer, corresponding to the initial phase of mineralization. It seems that age and infection-associated inflammation increase the burden of uterine dysfunction and lead to shell structural defects; however, the experimental evidence supporting this hypothesis is still lacking.

On the other hand, the alleviation of uterine inflammation by dietary modulation could be a potential strategy for improving ultrastructure characteristics and mechanical properties of shell, especially in the late phase period. Essential oils (EO), possessing antimicrobial and anti-inflammatory properties, have attracted great attention for their effectiveness in enhancing eggshell quality (Abou-Elkhair et al., 2018; Feng et al., 2021). Dietary EO supplementation could elevate shell thickness and improve ultrastructure, thus increasing breaking strength, which may be linked to the beneficial effects of EO on uterine function restoration (Abdel-Wareth and Lohakare, 2020; Zhao et al., 2021). Essential oils (including carvacrol, thymol and eugenol or trans-cinnamaldehyde) can prevent the colonization and infection of *Salmonella Enteritidis* in the layer reproductive tract (Upadhyaya et al., 2013, 2015). Furthermore, numerous plant extracts have been widely used to mitigate inflammatory diseases such as endometritis in humans and cows (Lv et al., 2015; Mandhwani et al., 2017), confirming its effectiveness in relieving uterine inflammation. Although it has been reported that EO could enhance shell mechanical properties presumably associated with the improvements of ultrastructure, it is still not known whether EO could recover uterine functions in biomineralization by ameliorating its inflammatory status.

In the present study, we hypothesized that inflammatory status is a crucial driver of uterine function regarding eggshell

ultrastructure formation. We initially investigated the effects of lipopolysaccharide (LPS)-induced uterine inflammation on ultrastructure and mechanical properties of eggshell, and the expression of matrix proteins and ionic transporters during eggshell formation. Then, age-related inflammation was reversed by dietary EO supplementation and the modulatory effects on uterine functions, structure characteristics and quality of eggshell were assessed. This study provides insight into the mechanism underlying inflammation-associated deteriorations in ultrastructure and mechanical properties of eggshell, which may contribute to future research on improving eggshell quality in the late phase of laying hens.

2. Materials and methods

2.1. Animal ethics statement

The animal protocols for this study were approved by the Animal Care and Use Committee of Feed Research Institute of the Chinese Academy of Agriculture Sciences. All animal experiments were performed in accordance with the ARRIVE guidelines (Kilkenny et al., 2012).

2.2. Experimental design

In the first trial, a total of 72 healthy Hy-line Brown laying hens at 36 wk of age were randomly allocated into 3 groups and raised in individual cages. After 1-wk acclimation, the injection programs were conducted as previously reported (Bedrani et al., 2013). In brief, the control birds were intravenously injected with phosphate buffered saline (PBS), or injected with PBS containing LPS from *Escherichia coli* O55:B5 (Sigma–Aldrich, St. Louis, MO, USA) with doses of 1 mg/kg of body weight (BW; single injection group, SI), or injected with LPS at 24 h intervals (multiple injection group, MI): 1 mg/kg of BW on d 0 and 0.5 mg/kg of BW on d 1 and 2.

In the second trial, a total of 288 Hy-line Brown laying hens at 60 wk of age were randomly divided into 4 groups with 8 replicates of 9 birds each. Before this experiment, all hens were fed with the basal diet for 1-wk adaption. Birds received the basal diet (control) or the basal diets supplemented with 50, 100 and 200 mg/kg mixed EO (M50, M100 and M200). The trial lasted 12 wk. The EO product was kindly provided by Beijing Feeding Feed Sci. & Tec. Co., Ltd. (Beijing, China) with carvacrol, thymol and cinnamaldehyde as active ingredients. The concentration of carvacrol, thymol and cinnamaldehyde in EO determined by high-performance liquid chromatography (HPLC) was 3.01%, 3.01% and 12.73%, respectively. Birds were raised in cages with 3 birds in each cage and at the end of the trial, one bird from each replicate was reared in an individual cage for determining oviposition time and sampling. All hens used in this trial remained in good health and medical intervention was not applied to any birds.

The basal diet (Table S1) used in this study was formulated according to NRC (1994) recommendations and China Feeding Standard of Chicken (NY/T 33–2004). All birds in this study had free access to the feed and water in 3-tier cages, with the cage size: 45 cm \times 45 cm \times 45 cm. Room temperature was maintained at approximately 24 °C and the lighting schedule was 16 h per day.

2.3. Sample collection

Each cage was equipped with an automatic monitoring control system (Registration No. of Computer Software Copyright: 2015SR228983) to monitor the oviposition behavior. When an egg was laid, the system would record the time. Then, the oviposition period (the total time an egg spent in the oviduct)

could be calculated and the time corresponding to the initial phase of shell formation could be predicted. In trial 1, the injection process was carried out 30 min after oviposition and the uterine tissues were collected 8 h after injection (Bedrani et al., 2013). In agreement with previous studies, our pretest indicated that the injection with LPS at 1 mg/kg BW could stimulate the expression of proinflammatory cytokines in the uterus of laying hens. Seven days after the first LPS challenge, 8 birds from each group were sacrificed at 8.5 h post oviposition (the initiation stage of eggshell formation). Duodenum mucosa and mucosa of uterine tissues surrounding the eggs were collected and snap frozen immediately in liquid nitrogen and then stored at -80°C until further analysis. The remaining portion of the uterus was isolated and then fixed in 4% paraformaldehyde solution for 24 h for histological analysis. The blood was collected and serum sample was separated by centrifugation at $2,740 \times g$ at 4°C for 10 min and then stored at -80°C for further analysis. Following multiple injection treatment, there were no complete eggs produced before d 5 and then 10 eggs each day were collected from the control and MI groups.

In trial 2, a total of 10 egg samples (5 eggs/replicate per d \times 2 d) from each replicate were collected over 2 successive days at the end of wk 0, 4, 8 and 12. Since the beneficial effects regarding eggshell quality were maximized at medium dosage (100 mg/kg) of EO addition, one bird from each replicate was randomly selected from the control and M100 groups for blood, intestine and uterus sample collection at the end of the feeding trial. The sample treatment was the same as that in trial 1.

2.4. Eggshell physical and mechanical properties

Eggshell thickness and breaking strength were measured by the Egg Shell Gauge and Egg Force Reader (Israel Orka Food Technology Ltd., Ramat Hasharon, Israel), respectively. TMS-Pro texture measurement system (Food Technology Corporation, Virginia, USA) was used to determine the stiffness of eggshell and then the elastic modules and fracture toughness of each egg were calculated as previously described (Mabe et al., 2003; Zhang et al., 2017). After removing egg contents, eggshell was washed, air-dried at room temperature, and weighed. Eggshell ratio was calculated as eggshell weight/egg weight \times 100.

2.5. Chemical components in eggshell, serum and uterus

The extraction and measurement of organic matrix proteins in eggshell were performed according to previous methods (Ahmed et al., 2005; Feng et al., 2020). Approximately 500 mg eggshell powder was weighed and demineralized with 20% acetic acid. They were mixed with distilled water (1:1, vol:vol), freeze-dried, dissolved in an extraction milieu and then dialyzed (cutoff 3,500 Da). The supernatant of the extraction was used to determine protein concentration (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Approximately 300 mg uterine tissue was homogenized and weighed. Eggshell powder and uterine samples were dissolved in HNO_3 and H_2O_2 solution (1:1, vol:vol) and then digested by the microwave dissolution instrument (MDS-10, Shanghai Xinyi Instrument Technology Co., Ltd., Shanghai, China). Flame atomic absorption spectrophotometry (Zeenit700 P, Analytik Jena, Germany) and a spectrophotometer (UV-2700, Shimadzu, Japan) were used to determine calcium and phosphorus contents in samples, respectively. Serum contents of calcium and phosphorus were detected with reagents following the instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.6. Eggshell ultrastructure

Shell pieces from the equator were collected with the assistance of tweezers, which was performed carefully to keep fracture surface smooth and the structure integrity for observation of the ultrastructure. The ultrastructure of the external shell surface and the cross section from the equatorial section of each egg sample was evaluated using a scanning electronic microscope (FEI Quanta 600, Thermo Fisher Scientific Ltd., Portland, OR, USA). Eggshell samples used for observing the ultrastructural variations in mammillary layer were prepared as previously described (Bain, 1990; Feng et al., 2020). Mammillary thickness, effective thickness (including palisade, vertical crystal layer and cuticle) and mammillary knob width were measured and calculated with a ruler of scanning electronic microscope. Regarding the variations in the mammillary layer, each variant was assigned a score according to its incidence in eggshell and the total score reflecting the overall variation degree of eggshell ultrastructure was calculated by the sum of all variant scores. Five egg samples from each replicate and one piece per egg were randomly selected for scanning electronic microscope and triplicate measures were performed on each piece.

2.7. Uterine histomorphology

Uterus tissue samples were fixed with 4% paraformaldehyde solution for 24 h and then embedded in paraffin. The paraffin samples were cut into 5 μm thick sections and then were stained with hematoxylin and eosin for histomorphology analysis. The villus length, height, width and area of mucosal folds, and ratio of edema or dissolution of tubular glands were assessed as previously described (de Moraes et al., 2021; Ma et al., 2020) using a light microscope coupled with image-processing software (Image J 1.53). The villus length of the uterus was measured from the top of the villus to the top of the lamina propria. The height of mucosal folds was defined as the vertical length from the epithelium to the fold, and the width of mucosal folds was measured by drawing a perpendicular line across the widest part of the mucosal folds. The outline of mucosal folds was traced and their areas were measured. The measurements were carried out on 8 replicates per group, with 6 measured values for each sample.

2.8. RNA isolation and real-time quantitative PCR

Total RNA in the duodenum and uterine tissues was isolated using EasyPure RNA kit (Beijing Transgene Biotech Ltd., Beijing, China) following the manufacturer's instructions. The purity and concentration of extracted RNA were measured by an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., VT, USA). RNA samples were reverse-transcribed using EasyScript First-Strand DNA Synthesis Super Mix (Beijing Transgene Biotech Ltd., Beijing, China) according to the manufacturer's procedures. Real-time PCR for determining the gene expression was performed on the Applied Biosystems 7500 real time PCR System with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, MA, USA). The reaction procedures were as follows: 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min. Primer sequences used in this study are listed in Table S2. The size of all amplified products was confirmed by electrophoresis on a 1.5% (wt:vol) agarose gel with gelred (SolarGelRed Nucleic Acid Gel Stain, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and visualized in Gel Doc XR + System (Bio-rad laboratories, Inc., CA, USA). The measurements were performed in duplicate and the relative mRNA expression levels were normalized to β -actin by the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001).

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and differences were assessed with Duncan's Multiple Range Test using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA). Regression analysis was employed to evaluate the linear and quadratic effects of dietary EO addition. A Student's *t*-test was used to analyze serum and uterus chemical composition, RT-qPCR, and uterus histology in the second trial. Data were expressed as the mean with their pooled standard error of the mean (SEM) and values were considered significant at a *P*-value < 0.05.

3. Results

3.1. Eggshell quality deteriorated following LPS challenge

There were no complete eggs collected before d 5 due to a temporary cessation of oviposition in response to LPS treatment. This could be attributed to intense inflammatory stress induced by multiple injections of LPS. The effects of LPS injection on eggshell quality were assessed on eggs collected within the following days (d 5 to 14 after injection; Fig. 1). Shell ratio, shell thickness and the mechanical properties of eggshell (breaking strength, stiffness, fracture toughness and elastic modules) declined significantly after

treatment and then recovered to normal levels about 12 d after injection (Fig. 1D–I). The weight of egg and eggshell showed a similar trend whereas shape index changed largely (Fig. 1A–C).

3.2. LPS challenge altered eggshell ultrastructure and chemical components of eggshell, serum and uterus

Hierarchical cluster analysis based on egg and eggshell attributes in the multiple LPS injection group showed 3 main clusters (Fig. 2A). The first one (M1) contained egg samples collected from 5 to 8 d after injection, the second cluster (M2) from 9 to 12 d after injection and the third (M3) from 13 to 14 d after injection. Then, eggshell samples from these 3 clusters were used to further analyze their chemical components and ultrastructure characteristics.

Mammillary caps in the control were round and have the normal honeycomb appearance (Fig. 2B, F and G), which provided a large area of contact between the shell and the outer shell membranes and the fiber tracks were deeply etched into the caps, indicating a strong association of membrane fibers and the initial calcium carbonate crystals. However, weak bonds between outer shell membrane and mammillary caps were observed in eggshell from the M1 and M2 groups (Fig. 2C and D). In addition, the structure of mammillary knobs was destroyed due to LPS treatment and some knobs even lacked normal basal caps (Fig. 2H and I). The

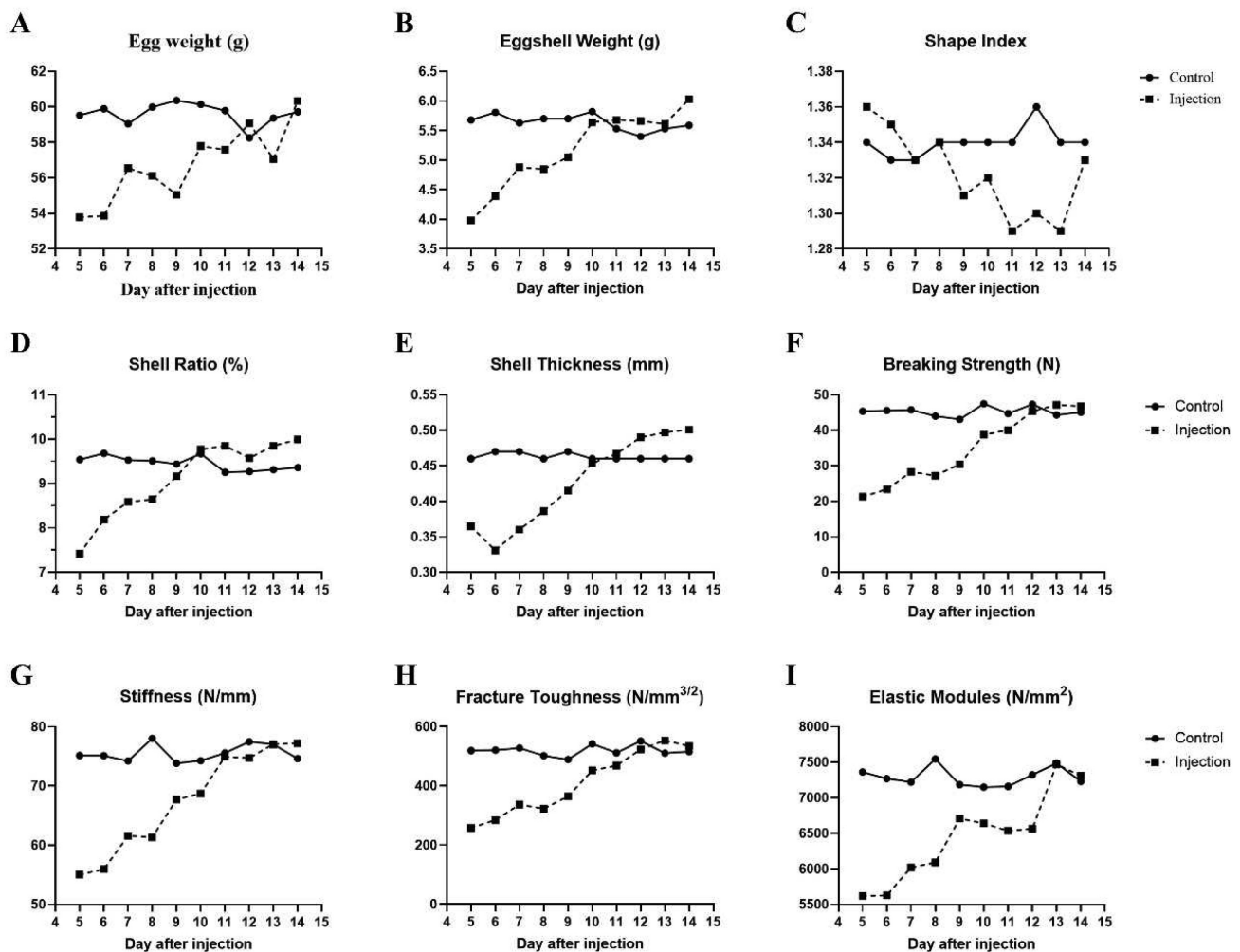


Fig. 1. Lipopolysaccharide (LPS) challenge induced deteriorations in eggshell quality. Evolution of egg weight (A), eggshell weight (B), shape index (C), shell ratio (D), shell thickness (E), breaking strength (F), stiffness (G), fracture toughness (H) and elastic modules (I) of eggshell from the control and LPS injection groups.

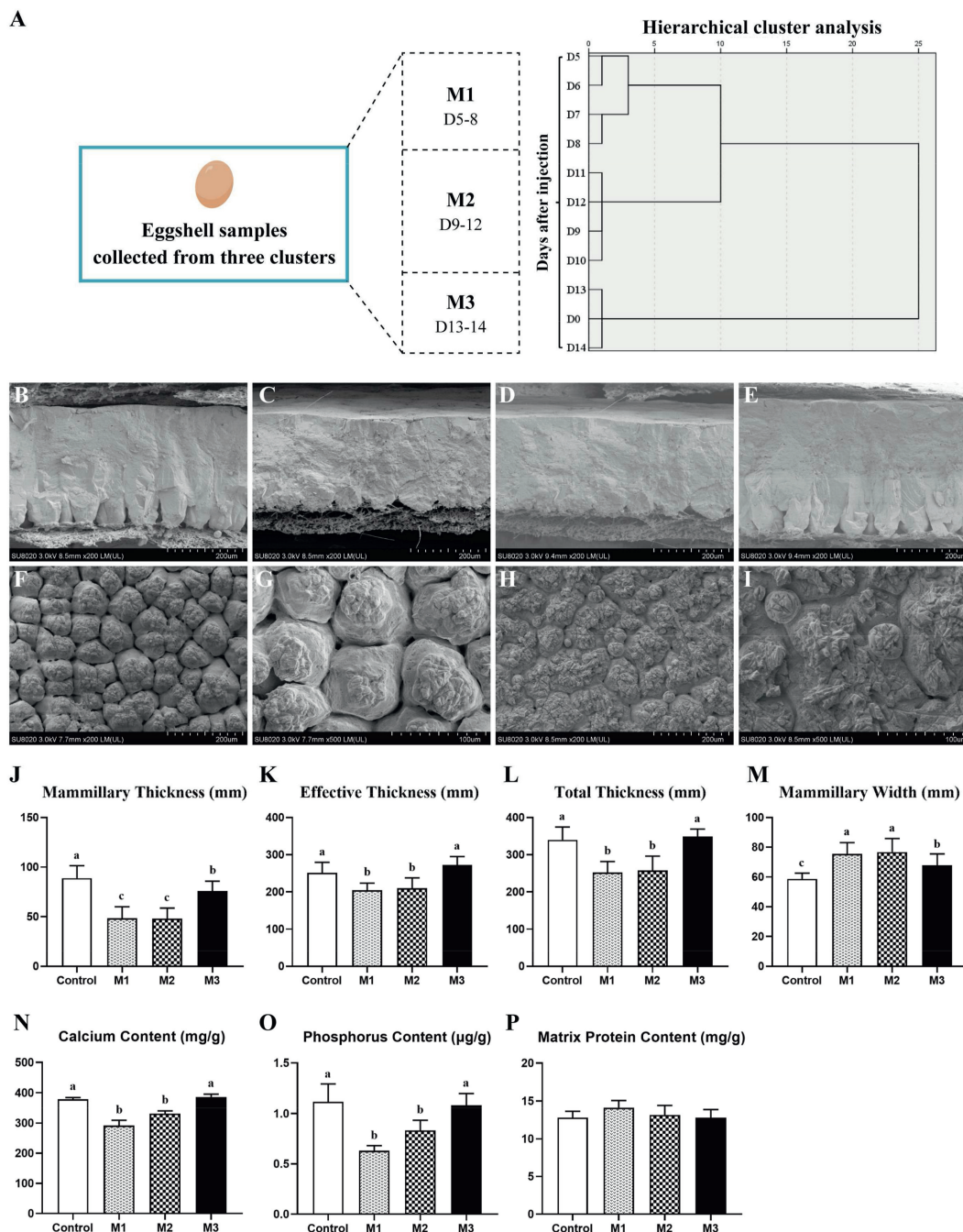


Fig. 2. Lipopolysaccharide (LPS) challenge modified ultrastructure characteristics and chemical composition of eggshell. (A) Hierarchical cluster analysis of eggshell quality attributes during the post-LPS challenge period. Control means eggshell samples from control laying hens without LPS challenge; M1, M2 and M3 mean eggshell samples in multiple-injection groups were divided into 3 clusters, including M1 (d 5 to 8), M2 (d 9 to 12) and M3 (d 13 to 14). Ultrastructure observation (magnification 200×) of eggshell from the control (B), M1 (C), M2 (D) and M3 (E) groups. Ultrastructure observation in the mammillary layer of eggshell from control (F, magnification 200×; G, magnification 500×) and M1 (H, magnification 200×; I, magnification 500×) groups. Ultrastructure parameters, including mammillary thickness (J), effective thickness (K), total thickness (L) and mammillary width (M) of eggshell collected from laying hens following LPS challenge. The contents of calcium (N), phosphorus (O) and matrix protein (P) in eggshell collected from laying hens following LPS challenge. Values are presented as mean ± SD, n = 8. ^{a-c} Means with different letters indicate a significant difference (P < 0.05).

size of mammillary knobs was not even and type B bodies occasionally occurred in the mammillary layer. The junctions between mammillary bodies could not be discriminated, though the organic membrane had been removed by the chemical method.

Mammillary thickness, effective thickness and total thickness of eggshell from the M1 and M2 groups were significantly lower (P < 0.05) than those from the control and M3 groups, while the width of mammillary knobs in the M1 and M2 groups were

significantly higher (P < 0.05) than those in the control and M3 groups (Fig. 2J–M).

Calcium and phosphorus contents of eggshell in the M1 and M2 groups were lower (P < 0.05) than those in the control and M3 groups, while matrix protein contents of eggshell were not affected (P > 0.05) by treatments (Fig. 2N–P). Besides, compared to the control, serum calcium and phosphorus contents were significantly decreased (P < 0.05) in the SI group and uterus calcium and

phosphorus contents were significantly decreased ($P < 0.05$) in both the SI and MI groups (Fig. S1).

3.3. LPS challenge induced uterine damage and inflammation

Single injection did not ($P > 0.05$) affect villus length or the height, width and area of mucosal folds (Fig. 3G–J), while signs of degeneration, necrocytosis or separation in mucosal epithelial layer

and edema of the tubular glands were observed (Fig. 3B and E). However, after multiple LPS injections, laying hens exhibited a shorter villus, and a lower height and area of mucosal folds in the uterus ($P < 0.05$) than the other groups (Fig. 3G, H and J). The apoptotic cells distributed in the mucosal epithelial layer and inflammatory cell infiltration in the sub-mucosa of the uterus were observed in the MI group (Fig. 3C and F). Besides, single or multiple injections resulted in a higher ratio ($P < 0.05$) of edema or

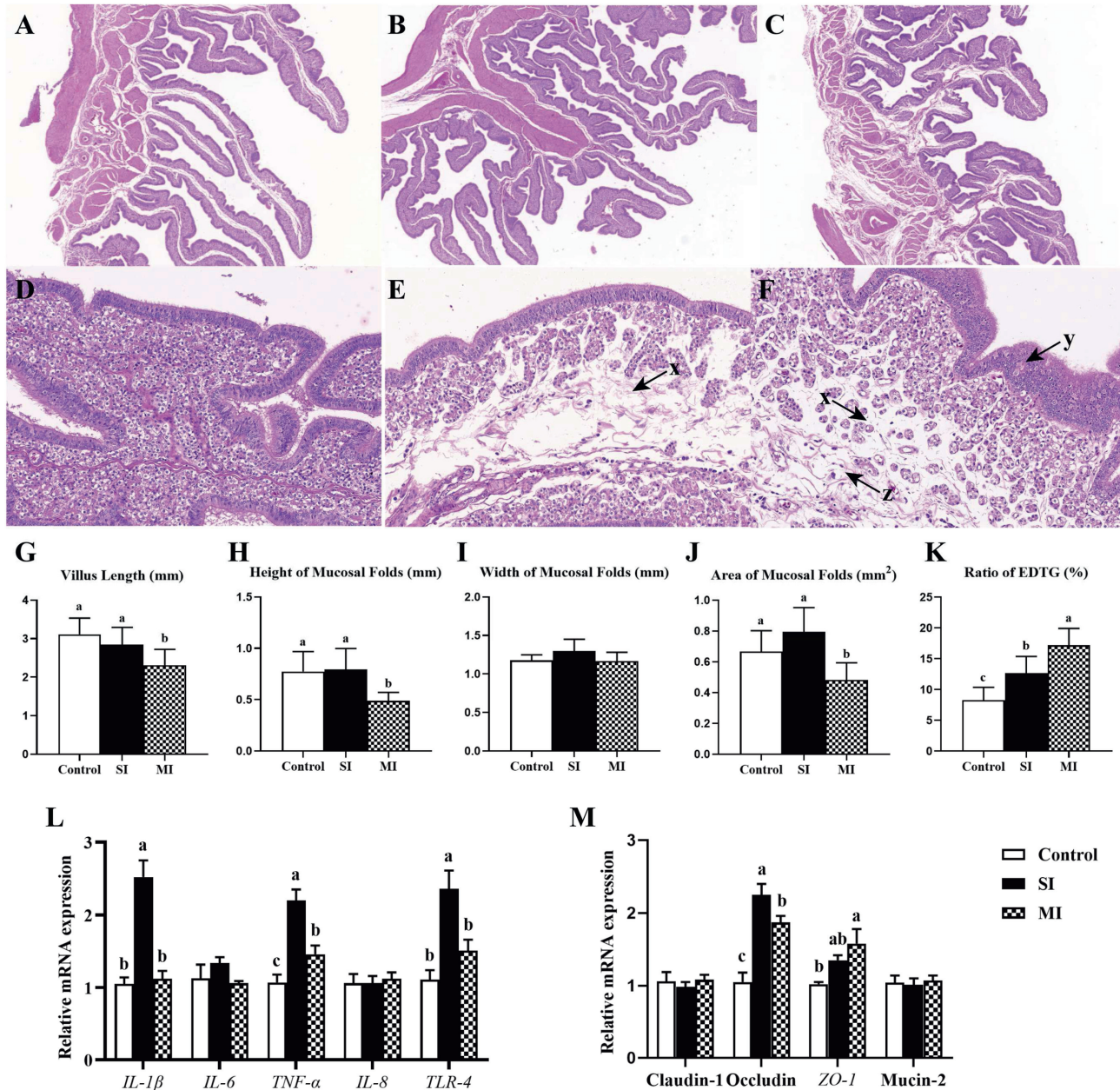


Fig. 3. Lipopolysaccharide (LPS) challenge induced uterine damage and altered mRNA expression of genes related to immune response and tight junction proteins in the uterus of laying hens. Morphological observation of the uterus from laying hens in the control (A, magnification 1.8 \times ; D, magnification 10 \times), SI (B, magnification 1.8 \times ; E, magnification 10 \times) and MI (C, magnification 1.8 \times ; F, magnification 10 \times) groups. SI means single injection with LPS 1 mg/kg body weight (BW); MI means multiple injection with LPS. Injection program: LPS injection with 24 h intervals, 1 mg/kg of BW on d 0 and 0.5 mg/kg of BW on d 1 and 2 at 3 h after oviposition. The lower-case letters in Fig. 3E and F, “x” means edema or dissolution of tubular glands, “y” means degeneration, necrosis or exfoliation of epithelial cells, “z” means lymphocytic infiltration. Morphology parameters included villus length (G), the height (H), width (I) and area (J) of mucosal folds, and ratio of EDTG (K) in uterus from laying hens following LPS challenge. EDTG = edema or dissolution of tubular glands. Values are presented as mean \pm SD, $n = 8$ (6 measured values for each sample). The villus length of the uterus was measured from the top of the villus to the top of the lamina propria. The height of mucosal folds was defined as the vertical length from the epithelial to the fold, and the width of mucosal folds was measured by drawing a perpendicular line across the widest part of mucosal folds. The outline of mucosal folds was traced and their areas were measured. The mRNA expression of immune response (L) and tight junction protein (M) related genes in uterus of LPS-challenged laying hens. ZO-1 = zonula occludens-1. Values are presented as mean \pm SEM, $n = 8$. ^{a-c} Means with different letters indicate a significant difference ($P < 0.05$).

dissolution of tubular glands in the sub-mucosa of the uterus than that in the control (Fig. 3K).

Multiple injections only stimulated the mRNA expression of *TNF- α* ($P < 0.05$), while single injection upregulated the expression of *IL-1 β* , *TNF- α* and *TLR-4* in uterine mucosa ($P < 0.05$) compared to those in the control (Fig. 3L). Relative mRNA expression of occludin and zonula occludens-1 in uterine mucosa of the MI group and occludin in the SI group was higher ($P < 0.05$) than those in the control (Fig. 3M).

3.4. LPS challenge modulated ionic transport and matrix protein synthesis in uterus

As for eggshell biomineralization-related genes, single injection upregulated the expression of ovotransferrin (*TF*) and ovalbumin (*OVAL*) in the uterine mucosa ($P < 0.05$) compared to those in the control (Fig. 4A). Birds in the multiple-injection group showed higher *OVAL* mRNA expression whereas lower osteopontin (*OPN*) mRNA expression ($P < 0.05$) in the uterine mucosa compared to those in the control. Injection treatment resulted in lower mRNA expression of *CALB1* ($P < 0.05$) in the uterine mucosa than those in the control (Fig. 4B).

3.5. Dietary EO addition regulated laying performance and egg quality in aged hens

Dietary EO addition had no significant effects ($P > 0.05$) on average egg weight, average daily feed intake and feed conversion ratio of laying hens during wk 1 to 4, wk 5 to 8, wk 9 to 12 and wk 1 to 12 of the experiment (Table 1). Egg production in the M50 group was higher ($P < 0.01$) than that in the control during wk 5 to 8 and wk 1 to 12 of the experiment. Additionally, there was a quadratic increase ($P < 0.05$) in egg production with the elevated addition of EO during wk 5 to 8. At the end of wk 8, albumen height and Haugh unit in the M50 group were higher ($P < 0.01$) than those in the control (Table 2). Haugh unit in the M50 and M200 groups was higher ($P < 0.01$) than that in the control, with a linear and quadratic elevation ($P < 0.05$) in Haugh unit following EO addition at the end of wk 12. Further, there was a linear increase ($P < 0.05$) in yolk color with the increasing addition of EO at the end of wk 8.

3.6. Dietary EO addition modified chemical composition and physical properties of eggshell

EO addition quadratically increased ($P < 0.05$) calcium content of eggshell, which was higher in the M100 group ($P < 0.05$) than in the control at the end of wk 8 and 12 (Table 3). Matrix protein

content of eggshell in the M100 group was lower ($P < 0.05$) than that in the control and M50 groups. Dietary EO supplementation elevated ($P < 0.05$) serum calcium content, while calcium content in uterus, and phosphorus content in the serum and uterus were not ($P > 0.05$) affected by dietary treatments (Fig. S2).

There was a linear and quadratic elevation ($P < 0.05$) in eggshell thickness following EO addition at the end of wk 4, 8 and 12. Eggshell index increased quadratically ($P < 0.05$) at the end of wk 4, 8 and 12 and increased linearly ($P < 0.01$) at the end of wk 12 with the elevated levels of EO in diets. A higher eggshell index ($P < 0.05$) was observed in the M50 group than that in the control at the end of wk 4 and 8. At the end of wk 12, EO supplementation led to a higher eggshell index ($P < 0.05$) compared to the control.

3.7. Dietary EO addition improved eggshell mechanical properties

Eggshell breaking strength was not affected ($P > 0.05$) by EO treatments at the end of wk 4, while a linear and quadratic increase ($P < 0.05$) at the end of wk 12 and a quadratic increase ($P < 0.05$) at the end of wk 8 were observed in eggshell breaking strength (Table 4). Compared to the control, dietary EO addition at 100 mg/kg increased ($P < 0.05$) eggshell strength at the end of wk 8 and 12, and EO addition at 50 mg/kg increased ($P < 0.05$) eggshell strength at the end of wk 8. There was a quadratic increase in eggshell stiffness in response to dietary EO addition at the end of wk 8 ($P < 0.05$) and wk 12 ($P < 0.01$). The inclusion of EO increased ($P < 0.05$) the stiffness of eggshell at the end of wk 8 and 12 (except in the M200 group at the end of wk 8). EO addition at the level of 100 mg/kg elevated ($P < 0.05$) fracture toughness of eggshell along with a quadratic increase ($P < 0.05$) at the end of wk 8. Elastic modules of eggshell were not ($P > 0.05$) affected by dietary treatments at any sampling times.

3.8. Dietary EO addition modulated ultrastructure characteristics of eggshell

Mammillary thickness, ratio of mammillary layer or ratio of effective layer, and the width of mammillary knobs were not ($P > 0.05$) affected by dietary EO supplementation (Fig. 5C, F, G and H). EO addition elevated ($P < 0.05$) the thickness of effective layer in eggshell (Fig. 5D), while total thickness of calcified layer in the M100 group was higher ($P < 0.05$) than that in the control (Fig. 5E). Effective thickness and total thickness of eggshell exhibited a linear and quadratic response ($P < 0.05$) with the increasing of dietary EO addition.

With regards to ultrastructural variants in the mammillary layer, there was a quadratic elevation in the frequency of early fusion

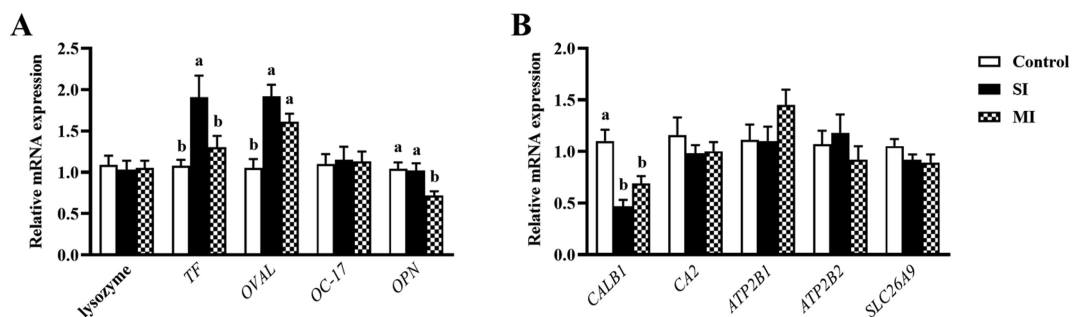


Fig. 4. Lipopolysaccharide (LPS) challenge altered the relative mRNA expression of key genes related to eggshell biomineralization in (A and B) the uterus of laying hens. SI means single injection with LPS 1 mg/kg body weight (BW); MI means multiple injection with LPS. Injection program: LPS injection with 24 h intervals, 1 mg/kg of BW on d 0 and 0.5 mg/kg of BW on d 1 and 2 at 3 h after oviposition. *TF* = ovotransferrin; *OVAL* = ovalbumin; *OC-17* = ovocleudin-17; *OPN* = osteopontin; *CALB1* = calbindin 1; *CA2* = carbonic anhydrase 2; *ATP2B1* = ATPase plasma membrane Ca^{2+} transporting 1; *ATP2B2* = ATPase plasma membrane Ca^{2+} transporting 2; *SLC26A9* = solute carrier family 26 member 9. Values are presented as mean \pm SEM, $n = 8$. ^{a, b} Means with different letters indicate a significant difference ($P < 0.05$).

Table 1
Effects of dietary essential oil supplementation on production performance in laying hens (60 to 72 wk).

Items	Treatments ¹				SEM	P-value		
	Control	M50	M100	M200		ANOVA	Linear	Quadratic
Egg production, eggs/hen per day								
wk 1 to 4	0.886	0.918	0.891	0.905	0.0072	0.422	0.644	0.835
wk 5 to 8	0.863 ^b	0.921 ^a	0.899 ^{ab}	0.900 ^{ab}	0.0066	0.009	0.208	0.046
wk 9 to 12	0.854	0.906	0.878	0.868	0.0071	0.051	0.954	0.171
wk 1 to 12	0.868 ^b	0.916 ^a	0.889 ^{ab}	0.889 ^{ab}	0.0060	0.032	0.650	0.206
Average egg weight, g								
wk 1 to 4	60.38	60.55	61.18	61.26	0.209	0.349	0.097	0.224
wk 5 to 8	60.57	61.20	61.70	61.68	0.212	0.201	0.066	0.096
wk 9 to 12	60.47	60.77	61.36	61.38	0.199	0.289	0.082	0.168
wk 1 to 12	60.46	60.84	61.41	61.44	0.197	0.226	0.062	0.120
Average daily feed intake, g/hen per day								
wk 1 to 4	111.66	111.17	110.94	109.29	0.651	0.625	0.188	0.413
wk 5 to 8	109.47	111.10	110.22	110.73	0.522	0.711	0.552	0.743
wk 9 to 12	109.86	110.29	110.59	109.60	0.633	0.952	0.851	0.844
wk 1 to 12	109.69	110.86	110.58	109.87	0.379	0.671	0.918	0.521
Feed conversion ratio, g/g								
wk 1 to 4	2.08	2.00	2.04	1.98	0.018	0.172	0.084	0.221
wk 5 to 8	2.10	1.97	1.99	2.00	0.020	0.090	0.221	0.086
wk 9 to 12	2.12	2.00	2.06	2.07	0.018	0.128	0.630	0.256
wk 1 to 12	2.09	1.99	2.03	2.01	0.016	0.105	0.197	0.167

SEM = standard error of the mean; ANOVA = analysis of variance. Values are presented as mean and SEM, *n* = 8.

^{a, b} Treatments with no common superscripts differ significantly (*P* < 0.05).

¹ Control means laying hens fed with basal diet; M50, M100 and M200 mean laying hens fed with basal diets supplemented with 50, 100 and 200 mg/kg essential oil, respectively.

Table 2
Effects of dietary essential oil supplementation on egg quality.

Items	Treatments ¹				SEM	P-value		
	Control	M50	M100	M200		ANOVA	Linear	Quadratic
Shape index								
wk 4	1.35	1.34	1.35	1.36	0.004	0.291	0.240	0.214
wk 8	1.35	1.35	1.36	1.35	0.003	0.564	0.716	0.772
wk 12	1.37	1.36	1.36	1.37	0.004	0.806	0.571	0.616
Albumen height, mm								
wk 4	6.13	6.63	6.28	6.22	0.105	0.365	0.814	0.568
wk 8	6.52 ^b	7.17 ^a	6.13 ^b	6.52 ^b	0.109	0.004	0.381	0.674
wk 12	6.92	7.24	7.08	7.10	0.057	0.272	0.199	0.199
Haugh unit								
wk 4	76.03	77.81	77.36	76.99	0.978	0.936	0.844	0.858
wk 8	79.46 ^b	84.02 ^a	76.48 ^b	79.50 ^b	0.785	0.003	0.388	0.664
wk 12	81.11 ^b	84.22 ^a	83.12 ^{ab}	83.60 ^a	0.401	0.029	0.012	0.018
Yolk color								
wk 4	5.65	5.95	5.85	5.63	0.097	0.601	0.694	0.456
wk 8	5.40	5.43	5.73	5.78	0.067	0.088	0.020	0.062
wk 12	4.85	4.75	4.63	4.69	0.060	0.617	0.234	0.495

SEM = standard error of the mean; ANOVA = analysis of variance. Values are presented as mean and SEM, *n* = 8.

^{a, b} Treatments with no common superscripts differ significantly (*P* < 0.05).

¹ Control means laying hens fed with basal diet; M50, M100 and M200 mean laying hens fed with basal diets supplemented with 50, 100 and 200 mg/kg essential oil, respectively

(*P* < 0.05) and cuffing (*P* < 0.01) along with a quadratic reduction (*P* < 0.05) in the incidence of type B mammillae in the mammillary layer with the increasing of EO addition (Fig. 5I–L and Table 5). As a result, total scores for mammillary variants decreased quadratically (*P* < 0.05) in response to EO addition and total scores in M100 were much lower (*P* < 0.05) than those in the control.

3.9. Dietary EO addition improved uterine morphology and alleviated uterine inflammation

Dietary EO supplementation did not (*P* > 0.05) affect the height, width and area of mucosal folds (Fig. 6F–H). Villus length was

elevated (*P* < 0.05) and the ratio of edema or dissolution of tubular glands tended to increase (*P* = 0.088) in response to EO supplementation (Fig. 6E and I). The inflammatory cell infiltration in the sub-mucosa of the uterus was observed in the control (Fig. 6A–D).

Dietary EO supplementation did not (*P* > 0.05) affect mRNA expression of *TNF-α*, *IL-8*, *IL-10* and *NF-κB* in the uterine mucosa, while *IL-1β* and *IL-6* mRNA levels were lower (*P* < 0.05) in the EO group than those in the control (Fig. 6J). The relative mRNA expression of genes related to tight junction proteins was not (*P* > 0.05) affected by dietary EO addition (Fig. 6K).

3.10. Dietary EO addition modulated ionic transport and matrix protein synthesis in the uterus

With regard to eggshell formation-associated genes, dietary EO supplementation resulted in lower mRNA levels (*P* < 0.05) of *OVAL* and *TF* (Fig. 7A), and higher mRNA levels (*P* < 0.05) of *CALB1*, ATPase plasma membrane Ca²⁺ transporting 1 (*ATP2B1*), ATPase plasma membrane Ca²⁺ transporting 2 (*ATP2B2*) and solute carrier family 26 member 9 (*SLC26A9*) in the uterine mucosa than those in the control (Fig. 7B).

4. Discussion

Inflammation is thought to be a critical driving force causing shell defects (Feng et al., 2020; Jiang et al., 2021; Nii et al., 2014), and it is unclear how uterine inflammatory status affects eggshell formation and quality. Here, we found that inflammation induced by LPS challenge interfered in matrix protein synthesis and ionic transport, indicated by upregulation of *OVAL* and *TF* and down-regulation of *CALB1*, thus destroying the ultrastructure and reducing the thickness of eggshell, thereby resulting in deterioration in mechanical properties. Dietary EO supplementation has been shown to improve eggshell quality, presumably associated with its anti-inflammatory and immunomodulatory activity (Feng et al., 2021; Valdivieso-Ugarte et al., 2019). In the current study, the attenuation of age-related uterine inflammation following

Table 3
Effects of dietary essential oil supplementation on chemical composition and physical properties of eggshell.

Items	Treatments ¹				SEM	P-value		
	Control	M50	M100	M200		ANOVA	Linear	Quadratic
Calcium content in eggshell, mg/g								
wk 4	328.69	335.73	340.28	325.21	2.608	0.164	0.516	0.078
wk 8	320.67 ^b	330.27 ^{ab}	338.62 ^a	328.48 ^{ab}	2.324	0.047	0.287	0.020
wk 12	324.56 ^b	334.64 ^{ab}	348.90 ^a	337.41 ^{ab}	2.816	0.016	0.100	0.009
Phosphorus content in eggshell, mg/g								
wk 4	1.08	1.15	1.04	1.10	0.025	0.544	0.904	0.979
wk 8	1.09	1.09	1.05	1.14	0.020	0.518	0.386	0.406
wk 12	1.15	1.09	1.24	1.15	0.026	0.265	0.687	0.724
Matrix protein content in eggshell, mg/g								
wk 4	13.03	12.93	13.04	12.83	0.157	0.963	0.685	0.908
wk 8	13.17	13.07	13.65	13.70	0.139	0.261	0.093	0.244
wk 12	13.04 ^a	13.03 ^a	12.31 ^b	12.76 ^{ab}	0.100	0.024	0.884	0.815
Eggshell thickness, × 10 ⁻² mm								
wk 4	42.31 ^b	45.34 ^a	45.11 ^a	44.69 ^a	0.305	<0.001	0.028	<0.001
wk 8	42.90 ^b	45.39 ^a	44.80 ^a	44.99 ^a	0.242	0.002	0.018	0.001
wk 12	42.17 ^b	44.64 ^a	44.56 ^a	44.53 ^a	0.256	<0.001	<0.001	<0.001
Eggshell weight, g								
wk 4	5.83	6.01	6.03	5.89	0.035	0.156	0.083	0.076
wk 8	5.58 ^b	5.88 ^a	5.84 ^{ab}	5.70 ^{ab}	0.045	0.046	0.683	0.047
wk 12	5.63 ^b	6.02 ^a	5.94 ^a	5.91 ^a	0.048	0.017	0.148	0.024
Eggshell index, %								
wk 4	9.48 ^b	9.85 ^a	9.79 ^a	9.64 ^{ab}	0.046	0.013	0.590	0.013
wk 8	9.29 ^b	9.80 ^a	9.62 ^{ab}	9.41 ^{ab}	0.056	0.007	0.900	0.007
wk 12	9.25 ^b	9.80 ^a	9.65 ^a	9.74 ^a	0.065	0.006	0.002	0.003

SEM = standard error of the mean; ANOVA = analysis of variance.

Values are presented as mean and SEM, n = 8.

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

¹ Control means laying hens fed with basal diet; M50, M100 and M200 mean laying hens fed with basal diets supplemented with 50, 100 and 200 mg/kg essential oil, respectively.

Table 4
Effects of dietary essential oil supplementation on mechanical properties of eggshell.

Items	Treatments ¹				SEM	P-value		
	Control	M50	M100	M200		ANOVA	Linear	Quadratic
Breaking strength, N								
wk 4	35.51	37.57	38.07	36.91	0.367	0.075	0.314	0.032
wk 8	34.11 ^b	36.78 ^a	36.71 ^a	35.31 ^{ab}	0.370	0.023	0.549	0.013
wk 12	33.98 ^b	36.12 ^{ab}	37.01 ^a	35.56 ^{ab}	0.391	0.038	0.025	0.027
Stiffness, N/mm								
wk 4	72.78	74.37	76.01	73.70	0.516	0.152	0.588	0.080
wk 8	72.66 ^b	75.93 ^a	76.01 ^a	73.70 ^{ab}	0.500	0.030	0.807	0.014
wk 12	69.35 ^b	74.45 ^a	73.57 ^a	72.97 ^a	0.572	0.013	0.113	0.007
Elastic modulus, N/mm ²								
wk 4	5095.20	4747.87	4929.22	4938.80	49.591	0.098	0.658	0.270
wk 8	5180.40	4841.42	4929.22	4938.80	57.371	0.230	0.307	0.188
wk 12	4920.19	4676.25	4643.24	4779.23	46.368	0.141	0.467	0.069
Fracture toughness, N/mm ^{3/2}								
wk 4	319.97	311.99	317.40	310.45	3.703	0.795	0.472	0.775
wk 8	294.56 ^b	309.12 ^{ab}	317.40 ^a	310.45 ^{ab}	3.360	0.034	0.126	0.041
wk 12	313.27	302.77	314.56	319.05	3.180	0.329	0.266	0.408

SEM = standard error of the mean; ANOVA = analysis of variance.

Values are presented as mean and SEM, n = 8.

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

¹ Control means laying hens fed with basal diet; M50, M100 and M200 mean laying hens fed with basal diets supplemented with 50, 100 and 200 mg/kg essential oil, respectively.

dietary EO intervention partly reversed dysfunction in biomineralization, as supported by the beneficial effects on ultrastructure and breaking strength of eggshell.

To explore how inflammation affects uterine functions in biomineralization, uterine inflammation of laying hens was induced by LPS challenge. In agreement with previous studies (Bedrani et al., 2013; Nii et al., 2011), an elevation in uterus *IL-1β* and *TNF-α* levels, along with lymphocytic infiltration and edema or dissolution of tubular glands was observed in challenged birds, suggesting the promotion of uterine inflammation as a result of LPS

treatment. Aging or pathogen infection gives rise to uterine inflammation, subsequently causing decreased calcium deposition and thinner shell thickness (Fan et al., 2014; Feng et al., 2020; Li et al., 2017; Nii et al., 2018; Qi et al., 2016). Similarly, in this study, uterine inflammation led to a sharp decrease in shell mechanical properties, as well as a dramatic reduction in the weight, ratio and thickness of eggshell. Inflammation may obstruct calcium transport and deposition in the uterus, which is critical for eggshell calcification, as supported by a decreased uterine calcium content and the suppressed expression of calcium transporter *CALB1* in the uterus.

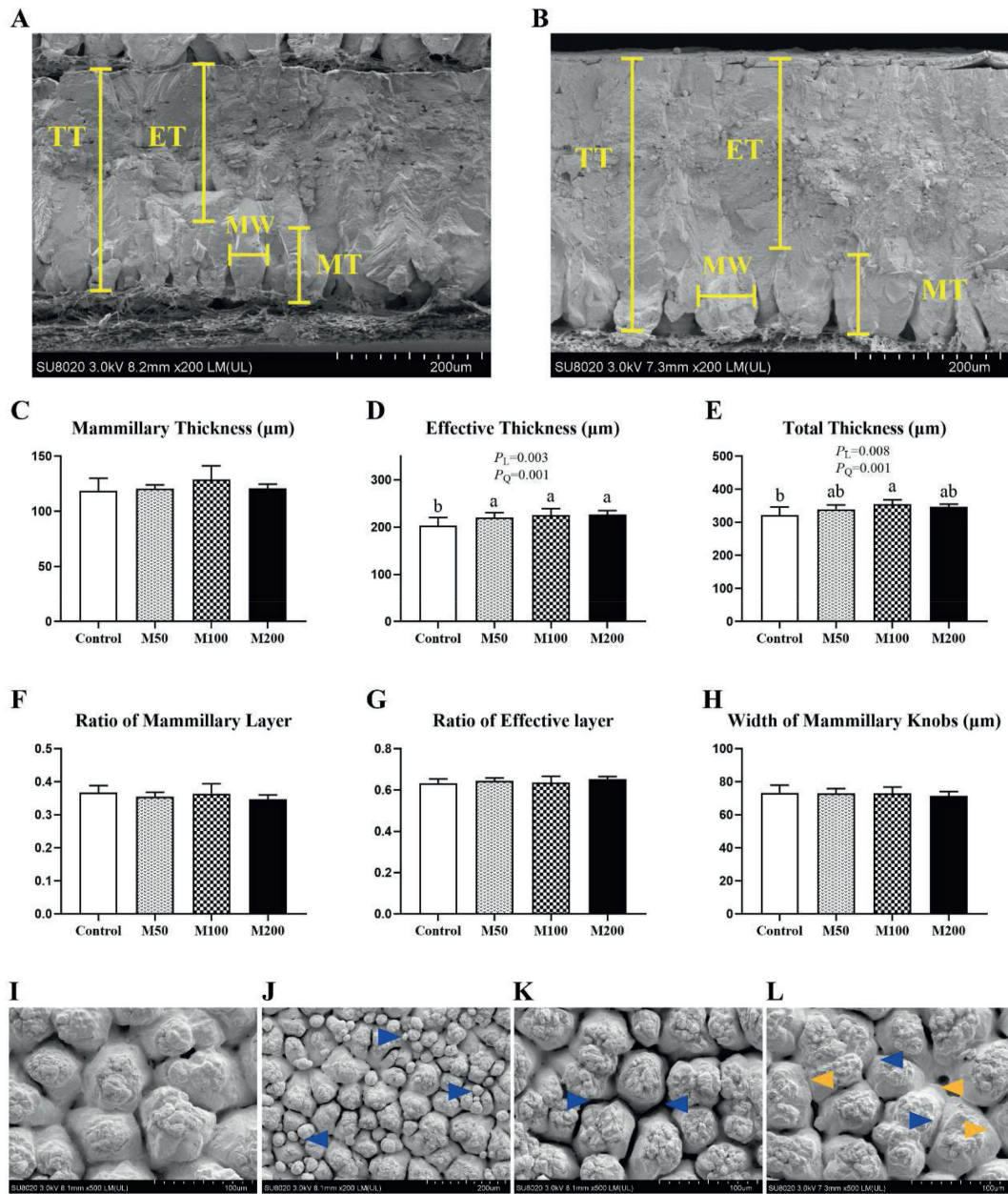


Fig. 5. Dietary essential oil supplementation modulated eggshell ultrastructure characteristics. Eggshell ultrastructure observation in the control (A) and essential oil-supplemented (B) groups. Effects of dietary essential oil supplementation on ultrastructure characteristics of eggshell (C–H). Control means laying hens fed with the basal diet; M50, M100 and M200 mean laying hens fed with the basal diets supplemented with 50, 100 and 200 mg/kg essential oil, respectively. Values are presented as mean ± SD, $n = 8$. ^{a, b} Means with different letters indicate a significant difference ($P < 0.05$). TT = total thickness; ET = effective thickness; MT = mammillary layer thickness; MW = width of mammillary knobs. Ultrastructural variations in the mammillary layer of eggshell. (I) Normal structure of mammillary knobs and (L) cuffing (yellow arrow) and early fusion of mammillary knobs (blue arrow; magnification 500×) in eggshell from essential oil-supplemented groups; (J) Type B mammillae (blue arrow; magnification 200×) and (K) late fusion of mammillary knobs (blue arrow; magnification 500×) in eggshell from the control.

Moreover, inflammation induced alterations in uterine morphology and barrier function, as evidenced by reduced villus length, height and area of mucosal folds, as well as the enhanced tight junctions of uterine mucosa. This may exert an adverse effect on the efficiency of ionic transport and supply in the uterus during eggshell formation, thereby decreasing calcium and phosphorus contents in eggshell.

The crucial role of eggshell ultrastructure has been well recognized in determining its mechanical properties (Athanasidou et al., 2018; Feng et al., 2020). In agreeance with previous reports (Li et al., 2017; Qi et al., 2016), virus infection-induced uterine

inflammation led to remarkable deteriorations in shell ultrastructure, characterized by extremely low thickness of mammillary and effective layers, and higher mammillary width. Moreover, we found obvious structural deteriorations in the mammillary layer, such as the impaired mammillary caps and a higher frequency of structural variations, including late fusion and type B mammillae. The effective layer (including palisade layer, vertical crystal layer and cuticle) is the major ultrastructural layer bearing external force (Radwan, 2015), and the internal organization in the mammillary layer and mammillae structural characteristics might play a decisive role in the structure and quality of the following layers (Dunn et al., 2012;

Table 5
Effects of dietary essential oil supplementation on ultrastructural variations in the mammillary layer of eggshell.

Items	Treatments ¹				SEM	P-value		
	Control	M50	M100	M200		ANOVA	Linear	Quadratic
Confluence	1.94	2.19	1.69	2.25	0.088	0.089	0.395	0.358
Type B	2.13	1.63	1.75	3.03	0.202	0.120	0.051	0.019
Type A	1.56	1.94	1.31	1.19	0.117	0.104	0.085	0.208
Early fusion	2.47 ^a	2.09 ^{ab}	1.72 ^b	2.25 ^{ab}	0.095	0.024	0.489	0.015
Late fusion	4.03	3.91	2.63	3.69	0.207	0.058	0.409	0.102
Changed membrane	2.19	2.25	1.75	2.44	0.182	0.609	0.712	0.581
Cuffing	2.31 ^a	1.75 ^{ab}	1.09 ^b	1.75 ^{ab}	0.137	0.009	0.159	0.006
Depression	1.16	1.16	1.13	1.03	0.032	0.475	0.125	0.281
Caps	2.66	2.59	1.97	2.66	0.131	0.178	0.875	0.210
Total score	20.44 ^a	19.50 ^{ab}	15.03 ^b	20.28 ^{ab}	0.697	0.017	0.813	0.022

SEM = standard error of the mean; ANOVA = analysis of variance.

Values are presented as mean and SEM, $n = 8$.

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

¹ Control means laying hens fed with basal diet; M50, M100 and M200 mean laying hens fed with basal diets supplemented with 50, 100 and 200 mg/kg essential oil, respectively.

Solomon, 2010). Late fusion of mammillae in response to uterine inflammation suggested a weaker contact between mammillary knobs, with the result that a fracture would propagate easily along the shell and outwards (Rodríguez-Navarro et al., 2015). Type B is a small spherical body, which does not make any direct contributions to the thickness and fracture resistance of the palisade layer (Gongruttananun, 2018). These structural variations in response to uterine inflammation imply impaired fusion of adjacent columns and a weak contact between the mammillary body and membrane fiber, in fact reflecting considerable modifications in the rate and orientation of crystal growth.

Actually, ultrastructure formation is precisely controlled by interactions between mineral and organic precursors, and matrix proteins exert a regulatory role in crystal size, morphology and mineralization kinetics (Athanasidou et al., 2018; Gautron et al., 2021). Ultrastructural variations may result from a disturbed regulation of matrix protein expression in response to inflammation. The current results showed that there was an elevated expression of *OVAL* and *TF* in the uterus following LPS challenge. Ovalbumin has been demonstrated to play a key role during the initial stage of eggshell formation, where it may serve as a stabilization agent for amorphous calcium carbonate, thus regulating calcification kinetics and guiding crystal growth to specific directions (Marie et al., 2015; Wolf et al., 2011). Ovotransferrin exhibited large modifications of the calcite morphology and crystal growth in vitro (Gautron et al., 2001). Therefore, the increased expression of *OVAL* and *TF* in response to uterine inflammation might indicate that calcite crystals would be driven to grow parallel to the c-axis rather than in all directions, presumptively contributing to the delay of mammillary fusion and the formation of type B mammillae. This was supported by the observation that age-related variations in mammillary structure were accompanied with higher concentrations of *OVAL* and *TF* in eggshell and upregulation of the *OVAL* gene in uterine mucosa (Feng et al., 2020; Panheleux et al., 2000). The *OVAL* gene has been demonstrated to show a high correlation with mammillae fusion rate and breaking strength of eggshell, through candidate gene association analysis (Duan et al., 2016). In addition, the synthesis of antibacterial proteins was regulated by immune status and age of laying hens (Javůrková et al., 2019; Lewko et al., 2021), such as inflammatory response in this study, possibly reflecting their immunological adaptation to fight against pathogens. Studies have pointed out that the expression of *OVAL* in the magnum of laying hens fulfils critical roles which have been recognized in immune defense against microbial infections in the oviduct (Nii et al., 2015; Zhao et al., 2016). In line with this view,

immune-defense proteins, like *OVAL*, *ABCA13* and β -defensins, were stimulated with uterine aging-inflammation (Feng et al., 2020). In the present study, LPS-induced inflammation might trigger host defense mechanisms to stimulate the expression of *OVAL* and *TF*, thereby leading to structural defects.

Inflammation-induced poor shell quality may be a consequence of uterine dysfunction in the synthesis of matrix proteins and transport of calcium. Age-related inflammation along with dysregulation of matrix protein in the uterus may conduce to shell ultrastructural deteriorations (Fathi et al., 2019; Feng et al., 2020). Thus, it is reasonable to infer that the alleviation of age-associated inflammation could be a potential target to optimize biomineralization and improve eggshell quality in the late laying period. Essential oil possesses anti-inflammatory and immunomodulatory properties with known beneficial effects on eggshell quality, which may be associated with promotion of uterine health (Abdel-Wareth and Lohakare, 2020; Feng et al., 2021). Dietary EO led to the mitigation of uterine inflammation in aged hens, as indicated by the decline of inflammatory cytokines such as *IL-1 β* and *IL-6* in uterine mucosa and alleviated histological damage. As a consequence, eggshell quality could be enhanced with EO supplementation, evidenced by the increased thickness, breaking strength and stiffness of eggshell. The increased shell thickness and weight may be partly associated with greater calcium absorption due to improved intestinal health and function (Abdel-Wareth and Lohakare, 2020; Feng et al., 2021), as evidenced by upregulated calcium transport and higher serum calcium levels. However, the attenuation of uterine inflammation failed to promote the trans-epithelial transport of calcium across the uterine plasma membrane into the cytoplasm, evidenced by no significant increase in uterine calcium content. It was reported that uterine injury, such as low gland density and damaged uterine morphology, due to long-term laying behavior would exacerbate uterine hypofunction involved in ion transport (Feng et al., 2020; Park and Sohn, 2018; Wistedt et al., 2019). In the current study, greater villus length along with mitigated inflammatory cell infiltration was observed in response to EO treatment, possibly favoring the efficient calcium transport and supply for calcification. In addition, the expression levels of ion transport *CALB1*, *ATP2B1*, *ATP2B2* and *SLC26A9* were elevated in the uterus of EO-fed birds. During eggshell biomineralization, intracellular calcium ions are transferred by *CALB1*, while *ATP2B1* and *ATP2B2*, the members of plasma membrane Ca^{2+} ATPase, are involved in the export of calcium into uterine fluid (Gautron et al., 2021; Sah et al., 2018). Another calcification material, bicarbonate, is actively extruded from uterine epithelial cells directly to

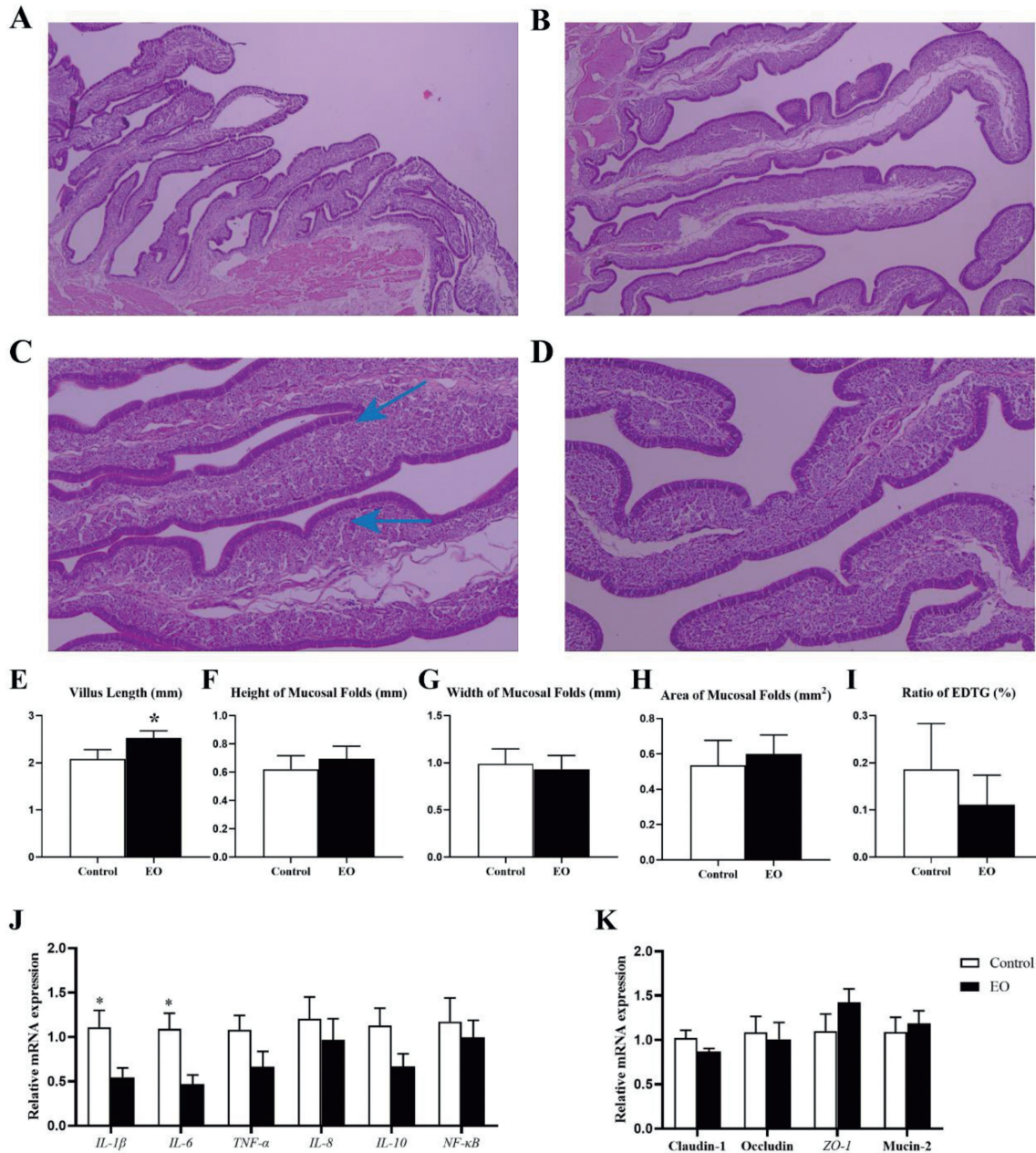


Fig. 6. Dietary essential oil supplementation modulated uterine morphology and the mRNA expression of genes related to immune response and tight junction proteins in uterus of laying hens. Morphological observation of the uterus from laying hens in the control (A and C) and EO (B and D) groups. Morphology parameters included villus length (E), the height (F), width (G) and area (H) of mucosal folds, and ratio of EDTG (I) in uterus of laying hens in the control and EO groups. The mRNA expression of immune response (J) and tight junction protein (K) related genes in uterus of laying hens in the control and EO groups. The villus length of uterus was measured from the top of the villus to the top of the lamina propria. The height of mucosal folds was defined as the vertical length from the epithelium to the fold, and the width of mucosal folds was measured by drawing a perpendicular line across the widest part of mucosal folds. The outline of mucosal folds was traced and their areas were measured. Control means laying hens fed with the basal diet. EO means laying hens fed with the basal diet supplemented with 100 mg/kg essential oil. EDTG = edema or dissolution of tubular glands. ZO-1 = zonula occludens-1. Values are presented as mean \pm SD, $n = 8$ (6 measured values for each sample). Blue arrows indicate the inflammatory cell infiltration in the sub-mucosa of the uterus. Asterisks indicate a significant difference between groups ($P < 0.05$).

mineralization sites by the HCO₃⁻/Cl⁻-exchanger *SLC26A9* (Jonchère et al., 2012). In the present study, the upregulation of ion transport suggested the relief of inflammation could be favorable to the supply of sufficient calcium towards the mineralization front, subsequently resulting in higher calcium deposition and the elevated thickness of eggshell.

We next sought to explore the modifications of shell ultrastructure and uterine-related functions as a result of the alleviated uterine inflammation. In the current study, EO intervention

relieved these adverse effects on ultrastructural traits, as exemplified by the increased thickness of the mammillary layer and whole shell, and the increased incidence of early fusion and cuffing structure. These outcomes indicated that mitigation of uterine inflammation led to favorable structure variations, assisting in its overall resistance to external forces and the prevention of cracks from propagating through the shell and outwards from the force points. The organic matrix has been demonstrated to play a crucial role in modulating ultrastructure formation (Gautron et al., 2021)

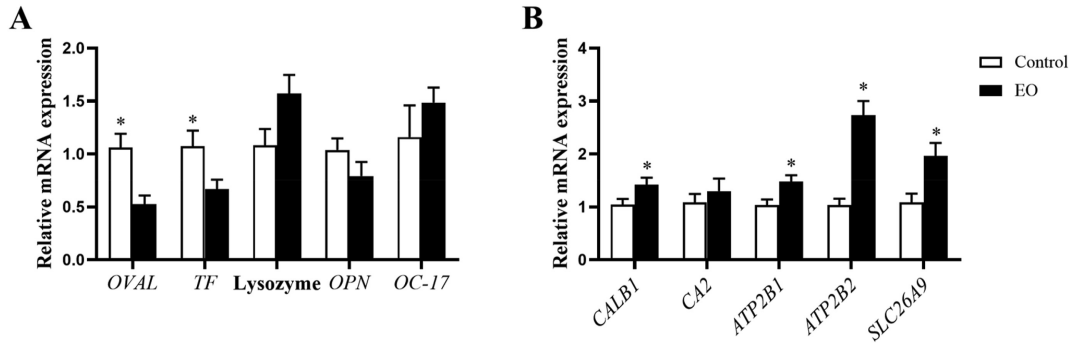


Fig. 7. Dietary essential oil supplementation regulated the mRNA expression of key genes related to eggshell biominerzation (A and B) in the uterus of laying hens. Control means laying hens fed with the basal diet. EO means laying hens fed with the basal diet supplemented with 100 mg/kg essential oil. OVAL = ovalbumin; TF = ovotransferrin; OPN = osteopontin; OC-17 = ovocleidin-17; CALB1 = calbindin 1; CA2 = carbonic anhydrase 2; ATP2B1 = ATPase plasma membrane Ca²⁺ transporting 1; ATP2B2 = ATPase plasma membrane Ca²⁺ transporting 2; SLC26A9 = solute carrier family 26 member 9. Values are presented as mean ± SEM, n = 8. Asterisks indicate a significant difference between groups (P < 0.05).

and we hypothesized that those structural variations were due to the alterations in matrix proteins and their regulatory effects. The remission of inflammation significantly downregulated the expression of matrix proteins OVAL and TF in the uterus of EO-fed birds. These modifications could be favorable for the assembly of mineral elements, and thus may contribute to structure and strength optimization. Of note, the synthesis of antibacterial proteins may reflect the status of the host defense system and their innate capacity to resist pathogens (Javůrková et al., 2019; Lewko et al., 2021). For instance, the upregulation of antibacterial proteins in inflamed endometrial tissue indicated their important role in uterine innate immunity against invading bacteria (Elhamouly et al., 2019; Ibrahim et al., 2016; Jonchère et al., 2010). The

alleviation of age-related inflammation may reduce the requirement for immune-defense proteins (such as TF and OVAL) against pathogenic infection in the uterus, consequently favoring the strength of shell ultrastructure and its mechanical properties. Similar findings were also observed in previous studies in which the expression of antibacterial proteins was suppressed in response to the reduction of inflammation in the reproductive tract (Hickey et al., 2011; Wigley et al., 2022). However, the complex regulatory network by which host physiological state affects matrix protein synthesis and their modulatory roles in biomineralization needs further investigation.

Collectively, our present study suggested that uterine inflammation-mediated dysfunction, especially in calcium

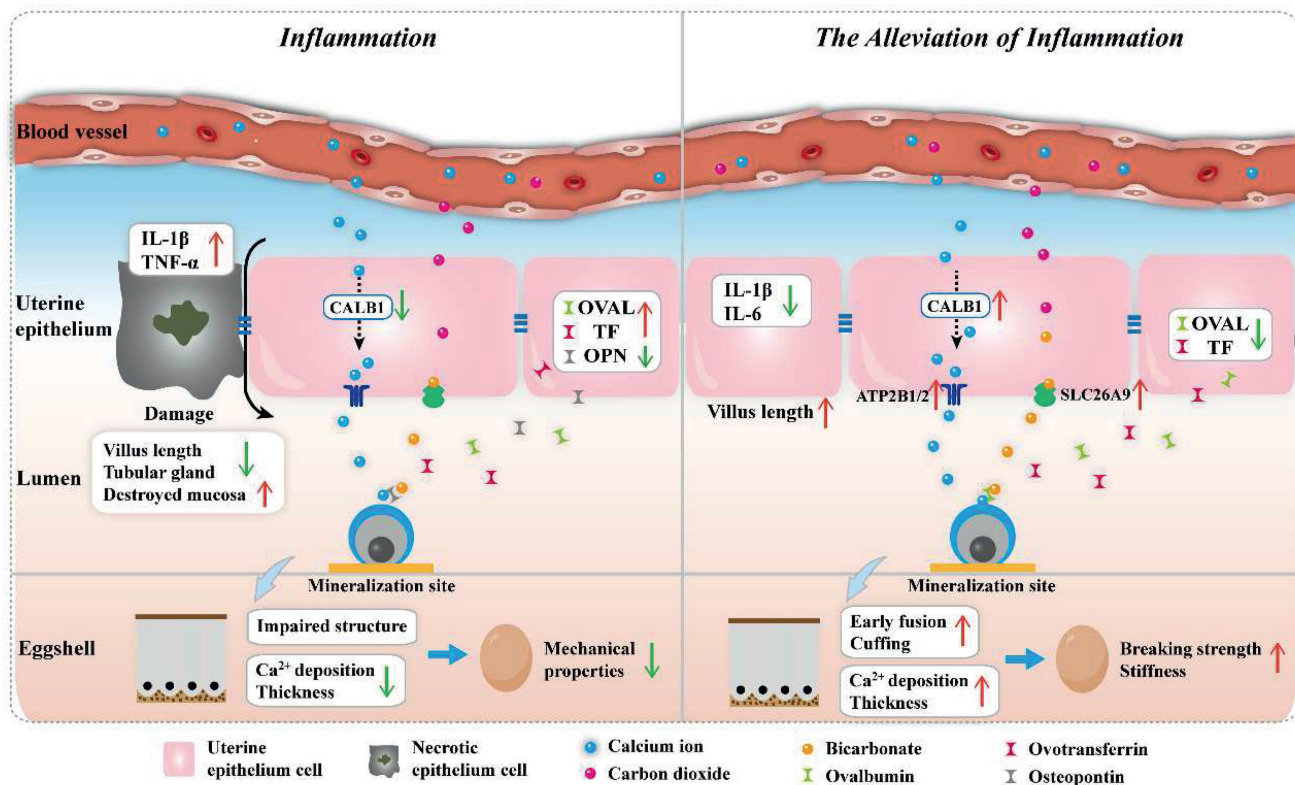


Fig. 8. A schematic model displaying the potential mechanism by uterine inflammation-mediated modification of eggshell quality. ↑Up arrows indicate the effects of stimulation; ↓down arrows indicate the effects of suppression. IL = interleukin; TNF = tumor necrosis factor; CALB1 = calbindin 1; OVAL = ovalbumin; TF = ovotransferrin; OPN = osteopontin.

transport and the synthesis of matrix proteins, may contribute to decreased thickness and damage to the ultrastructure of eggshell, thus compromising its mechanical properties. Conversely, the alleviation of uterine inflammation following dietary EO addition might also be involved in the enhancement of shell thickness and mammary layer structure, and the subsequent elevation in breaking strength and stiffness, by promoting calcium and bicarbonate transport and suppressing the synthesis of *OVAL* and *TF*. These findings are summarized in Fig. 8. Taken together, uterine inflammatory state may modulate uterine functions in biomineralization, especially calcium transport and the synthesis of matrix proteins such as *OVAL* and *TF*, thereby determining calcium deposition and ultrastructure characteristics, and subsequent mechanical properties of eggshell.

5. Conclusion

In summary, alterations in uterine inflammatory state modulated eggshell thickness and ultrastructure characteristics, and their subsequent impacts on mechanical properties implied a critical role of inflammatory status in mediating the effects on uterine functions involved in shell biomineralization. These modifications in uterine functions are believed to primarily affect calcium transport and the synthesis of matrix proteins, especially such as *OVAL* and *TF*, which in turn modulate calcium precipitation and ultrastructure formation, thereby determining the mechanical properties of eggshell. These findings provide a novel insight into uterine inflammation-mediated modification of eggshell quality and will facilitate the exploration of preventive strategies for declines in shell quality in the late laying period.

Author contributions

Jia Feng: formal analysis, data curation, writing-original draft, conceptualization. **Mingyuan Lu:** formal analysis, investigation. **Lingling Ma:** formal analysis. **Haijun Zhang:** methodology, conceptualization. **Shugeng Wu:** conceptualization. **Kai Qiu:** conceptualization. **Yuna Min:** methodology, resources. **Guanghai Qi:** conceptualization, supervision. **Jing Wang:** supervision, writing-review & editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

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