Dietary oregano essential oil supplementation improves intestinal functions and alters gut microbiota in late-phase laying hens

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Abstract

**Background:** Dietary essential oil (EO) supplementation can exert favorable effects on gut health in broilers. However, it is unknown whether EO could improve intestinal functions, consequently beneficial for egg performance and quality in late-phase laying hens. This study was aimed to investigate the potential effects of EO on production performance, egg quality, intestinal health and ileal microbiota of hens in the late phase of production. A total of 288 60-week-old Hy-line Brown laying hens were randomly divided into 4 groups and fed a basal diet (control) or basal diets supplemented with oregano EO at 100, 200 and 400 mg/kg (EO100, EO200 and EO400).

**Results:** Dietary EO supplementation resulted in a quadratic decrease ($P < 0.05$) in feed conversion ratio with lower ($P < 0.05$) feed conversion ratio in EO200 group than the control during weeks 9–12 and 1–12 of the trial. Compared to the control, EO addition resulted in higher ($P < 0.05$) eggshell thickness at the end of week 4, 8 and 12 and higher ($P < 0.05$) chymotrypsin activity. There was a quadratic elevation ($P < 0.05$) in ileal chymotrypsin and lipase activity, along with a linear increase in villus height to crypt depth ratio. Quadratic declines ($P < 0.05$) in mRNA expression of IL-1$\beta$, TNF-$\alpha$, IFN-$\gamma$ and TLR-4, concurrent with a linear and quadratic increase ($P < 0.05$) in ZO-1 expression were identified in the ileum with EO addition. These favorable effects were maximized at medium dosage (200 mg/kg) of EO addition and intestinal microbial composition in the control and EO200 groups were assessed. Dietary EO addition increased ($P < 0.05$) the abundances of Burkholderiales, Actinobacteria, Bifidobacteria, Enterococcaceae and Bacillaceae, whereas decreased Shigella abundance in the ileum.

**Conclusions:** Dietary EO addition could enhance digestive enzyme activity, improve gut morphology, epithelial barrier functions and modulate mucosal immune status by altering microbial composition, thus favoring feed efficiency and eggshell quality of late-phase laying hens.

**Keywords:** Essential oil, Feed efficiency, Immune status, Late-phase laying hen, Microbiota
Introduction
Declined laying performance and poor egg quality in the late laying period have seriously reduced the economic benefits, which are the key obstacles for extending laying period especially in late-phase laying hens [1]. The compromised intestinal functions, immune imbalance and intestinal flora disturbance due to high-intensity production are ascribed for the poor egg performance of hens in late laying period [2, 3]. Owing to the restriction of antibiotic utilization as growth promoters for animals, the use of natural bioactive compounds such as essential oils (EO) has attracted much attention to improve poultry health and performance [4].

EO are aromatic oily liquids extracted from plant material (flowers, buds, seeds, leaves, etc.), whose antibacterial properties have encouraged their usage as natural antibiotic alternatives for animal production [4]. The efficacy of EO on reducing the colonization of *Escherichia coli*, *Clostridium perfringens* and *Campylobacter jejuni* have been extensively investigated in broiler [5, 6] and pig [7, 8]. EO or their main components (such as thymol and carvacrol) can disturb the membrane structure and alter its permeability by partitioning the lipid fraction of plasma membrane, and thus exert antibacterial activity [4]. Furthermore, the beneficial effects of EO have been widely documented in poultry production. The supplementation of EO (containing thymol) in broiler diets may enhance growth performance, increase intestinal and pancreatic digestive enzyme activities [9, 10] and improve cellular and humoral immunity [11, 12]. A combined use of thymol and carvacrol was demonstrated to alleviate intestinal inflammation, the impaired intestinal integrity and barrier dysfunction induced by *C. perfringens* challenge in broilers [13]. Besides directly inhibiting the growth of pathogen bacteria, EO (blends of thymol and carvacrol or encapsulated cinnamaldehyde) could modulate intestinal microbial composition of birds [14, 15]. Thus, EO may exert a favorable effect on gut health via maintaining intestinal integrity and barrier functions, enhancing immune system activities and regulating gut microbiota. The inclusion of EO (thymol, carvacrol or menthol as active components) in layer diets was reported to improve laying performance and egg quality [16–18]. However, the effects of EO on the intestinal microbial community, mucosal barrier and immune status of laying hens in the late phase of production await further studies.

A great variety of plants are valued for their EO content and oregano (*Origanum vulgare L.*) are among the most widely used [4]. The main compounds of oregano EO are thymol, carvacrol, γ-terpinene and p-cimene, which exhibits some biological activities including antibacterial, anti-inflammatory and immune-regulating properties [7, 8]. In the present study, we hypothesized that dietary inclusion of oregano EO would positively alter the microbial composition, mucosal immune responses, and intestinal barrier functions, subsequently conducing to the improvements of egg performance and quality in laying hens. Therefore, this study was aimed to investigate the effects of dietary EO supplementation on laying performance, egg quality, gut morphology, microbial community, and relative mRNA expression of immune-related and tight junction-related genes in the intestine of laying hens in the late phase of production.

Materials and methods

Birds and experimental design
The animal protocols for this study were approved by the Animal Care and Use Committee of the Feed Research Institute of the Chinese Academy of Agricultural Sciences. A total of 288 60-week-old Hy-line Brown laying hens were randomly divided into 4 groups with 8 replicates of 9 birds each. The layers were commercially purchased from Xiaoming Agriculture and Animal Husbandry Co. Ltd. (Ningxia, China). Prior to the experiment, egg production and egg quality were assessed, which were similar across all the replicates. Birds received the basal diet without (Control) or with 100 mg/kg (EO100), 200 mg/kg (EO200) and 400 mg/kg (EO400) microencapsulated oregano EO product for 12 weeks. Oregano EO product (containing 5% oregano EO) was obtained from a commercial supply with carvacrol and thymol as active ingredients and calcium carbonate as carrier. The designed concentrations of oregano EO in the diets of EO100, EO200 and EO400 groups were 5, 10 and 20 mg/kg, respectively. The concentration of carvacrol and thymol in EO determined by high-performance liquid chromatography (HPLC) was ≥2.83% and ≥0.08%, respectively. All birds were housed in three-tier battery cages with 3 birds per cage (cage size: 45 cm × 45 cm × 45 cm) in an environmentally controlled house with temperature maintained at approximately 24 °C. All birds were fed with the same corn-soybean meal basal diet and provided with feed and water *ad libitum* with exposure to 16 h of light/d. The basal diet (Table 1) was formulated according to NRC (1994) recommendations. All hens remained in good health and medical intervention was not applied to any birds during the whole feeding period.

Sample collection
Five eggs per replicate were collected for egg quality determination every 4 weeks. Besides, one bird from each replicate was randomly selected at the end of weeks. 12 of the experiment and the intestinal tract was separated after slaughter. The middle portion of ileum were then isolated and approximately 1 cm segments of the midpoints of ileum were fixed in 10% neutral-buffered formalin for histological analysis. Ileal digesta were snap-frozen in liquid
nitrogen and stored at −80 °C for analysis of digestive enzyme activity and microbial composition. The mucosal samples of ileum were collected by gentle scraping the intestinal wall with glass microscope slides, snap-frozen in liquid nitrogen and stored at −80 °C for mRNA analysis.

### Laying performance and egg quality

Egg production and egg weight were recorded daily by replicate and feed consumption for each replicate was weighed every 4 weeks. Feed conversion ratio (FCR) was calculated as grams of feed consumption/egg weight for each replicate. Average daily feed intake and FCR were calculated every 4 weeks. As for egg quality measurement, each egg was individually weighed and egg height (mm) and width (mm) were recorded for shape index calculation (shape index = height/width). Breaking strength and the thickness of eggshell were measured by Egg Force Reader and Egg Shell Thickness Gauge (Israel Orka Food Technology Ltd., Ramat Hasharon, Israel). Haugh unit values, albumen height, and yolk color were measured by an Egg Analyzer (Israel Orka Food Technology Ltd., Ramat Hasharon, Israel). The cDNA samples were obtained by reverse transcription of the total RNA using the first-strand synthesis kit (TransGen Biotech Co., Ltd., Beijing, China). Real-time PCR for analysis of the gene expression was performed using SYBR Green (Thermo Fisher Scientific, MA, USA) on an ABI 6 flex real-time PCR instrument (Thermo Fisher Scientific, MA, USA). Primer sequences used in this study are shown in Table 2. The reaction conditions 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. Melt curve analysis was performed to confirm the PCR amplification specificity. Each sample was measured in duplicate and the relative mRNA expression levels were analyzed using 2^−ΔΔCt method [19].

### DNA extraction and analysis of ileal microbiota

The gut digesta samples (~200 mg of each sample) were used for microbial DNA extraction using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The quality of DNA samples was assessed by 1% agarose gel electrophoresis. The V3-V4 region of the 16S rRNA gene was amplified using the primer pair 338F/806R (5′-ACTCCTACGGGAGGCAGCA-3′ and 5′-GGACTACHVGGGTWTCTAAT-3′). PCR products were quantified with the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, USA). The Illumina platform was used to generate paired-end reads (2 × 300 bp). Sequencing and bioinformatics were performed on QIME2 platform of Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China) and the sequencing results were analyzed based on amplicon sequence variants (ASVs) [20]. Two obvious outliers in each group may interfere with the microbiota statistical analysis and thus were excluded in the following analysis. Alpha
Diversity indices (including Chao1 richness estimator, observed species, Shannon diversity index, and Simpson index) were calculated to evaluate microbial species evenness. Beta diversity was evaluated by principal coordinate analysis (PCoA) based on the unweighted UniFrac distance. Taxa abundances at the phylum, class, order, family and genus levels were statistically compared between groups. Linear discriminant analysis (LDA) combined effect size measurements (LEfSe) were used to identify the differences in microbial composition between groups. Pearson correlation analysis was conducted on the potential relationship between ileal gene expression levels, digestive enzyme activity, gut morphology, performance parameter and microbial composition.

The sequencing data have been deposited at the National Center of Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (accession number: PRJNA693086).

### Table 2 Sequences of real-time PCR primers

| Genes          | Primer sequence (5′→3′)                  | Accession no.       |
|----------------|------------------------------------------|---------------------|
| Claudin-1      | F: AAGTCGACATGAGGATGACCA                 | NM_00103611.2       |
|                | R: GCCACTGTGAGGATGACCA                   |                     |
| Occludin       | F: TCTACGGCTTCATCATCGTAC                | NM_05128.1          |
|                | R: TCTACGGCTTCATCATCGTAC                |                     |
| ZO-1           | F: TATGGAGGATCTGGCGGCGCTCC             | XM_015278981.1      |
|                | R: GAGGTGTCCATGCTAGTCC                 |                     |
| Mucin-2        | F: AGCGAGATGTGGCCGATG                  | NM_001318434.1      |
|                | R: AGGTGACACACACACACACACAC             |                     |
| IL-1β          | F: ACTGGGATCATAGGGTGCTA               | NM_204524           |
|                | R: GGTAGAAGTGAAGCGGTC                  |                     |
| IL-8           | F: GGCCTGATGGAGAATGA                  | AJ009800            |
|                | R: AGCTGACTCTGACTAGGAACCTG            |                     |
| IL-10          | F: CGCTGTCAAAGCTGCCTCGA            | NM_001004414.2      |
|                | R: CGTCTCTGATCGCTGCCTGATG            |                     |
| TNF-α          | F: GAGGCGTACTGCTGTC                  | NM_204267           |
|                | R: AGGCAAACACACACTGATGCCAC          |                     |
| IFN-γ          | F: AAACCGGCACATCAACACAC              | NM_00149.1          |
|                | R: GCCATCAGGAGGTTGTTTTC              |                     |
| TLR-4          | F: CCCTACAGGTGTTGTTGAC                | NM_001030693.1      |
|                | R: ACAGCTTCTAGCAGCGCAAT              |                     |
| β-actin        | F: GAGAAATGTTGGCGGATCACA            | L08165              |
|                | R: CCTGAACTCCTTCTGACCA              |                     |

F forward primer, R reverse primer

**Results**

**Laying performance and egg quality**

Dietary EO supplementation had no significant influences ($P > 0.05$) on egg production and average daily feed intake of laying hens during weeks 1–4, 5–8, 9–12 and weeks 1–12 of the experiment (Table 3). However, average egg weight increased ($P < 0.05$) linearly with the elevated levels of EO in diets during weeks 1–4, 5–8 and 1–12. There was a quadratic decrease ($P < 0.05$) in FCR with the increasing addition of EO during the whole experiment period. During weeks 9–12 and 1–12, dietary EO supplementation at 100 mg/kg decreased ($P < 0.05$) FCR of laying hens in comparison with the control. With regard to egg quality, there was no significant effects ($P > 0.05$) of dietary EO supplementation on eggshell strength, relative eggshell weight, shape index, albumen height, Haugh unit and yolk color at the end of week 4, 8 and 12 (Table 4). Eggshell thickness increased linearly and quadratically ($P < 0.05$) in response to the increasing addition of EO and eggshell thickness in EO supplemented groups were higher ($P < 0.05$) than that in the control at the end of week 4, 8 and 12.

**Intestinal morphology and digestive enzyme activity**

No significant changes ($P > 0.05$) were observed in ileal VH and CD of laying hens at the end of week 12 with the increasing EO supplementation, which, however, linearly elevated ($P < 0.05$) ileal VCR (Table 5). EO supplementation did not affect ($P > 0.05$) α-amylase activity in ileal digesta (Table 6). However, there was a quadratic elevation ($P < 0.05$) in ileal chymotrypsin activity along with a linear and quadratic increase ($P < 0.05$) in ileal lipase activity. Compared with the control, dietary EO inclusion at the level of 100 mg/kg increased ($P < 0.05$) ileal chymotrypsin activity.
Gene expression in the ileal tissue

No significant influences (P > 0.05) were observed on relative mRNA expression of claudin-1, occludin and mucin-2 in ileal mucosa of laying hens in response to the addition of EO (Fig. 1a). However, there was a linear and quadratic increase (P < 0.05) in relative mRNA expression of zonula occludens-1 (ZO-1) in the ileum with the increasing levels of EO in diets. Dietary supplementation with EO quadratically down-regulated (P < 0.05) the relative mRNA expression of interleukin-1 β (IL-1β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and toll-like receptor-4 (TLR-4) in the ileum by the increasing addition of EO (Fig. 1b).

Ileal microbial profile

No significant differences (P > 0.05) in species richness (as reflected by Chao1 and Observed_species indices) or alpha-diversity (as reflected by Shannon and Simpson indices) were observed in ileal microbiota at the taxonomic level (Fig. 2a). However, PCoA results based on the unweighted UniFrac distance showed separation of ileal microbial communities between control and EO-supplemented groups (Fig. 2b). Firmicutes and Proteobacteria were the dominant phyla in the control and EO-supplemented groups, account for more than 70% of the whole phyla (Fig. 3a). EO supplementation resulted in a decreased abundance of Firmicutes. The dominant classes were Bacilli and Clostridia, within Firmicutes across groups (Fig. 3b). Family analysis indicated that the abundance of Bifidobacteriaceae tended to increase (P = 0.060; Fig. 3c) with EO addition. At genus level, the Lactobacillaceae accounted for the greatest proportion of the microbiota (Fig. 3d). The abundance of Aeriscardovia and Aquabacterium abundances were increased (P = 0.064 and 0.018) with EO addition.

The LEfSe analysis was conducted to identify the relative richness (P < 0.05, LDA > 3.0; Fig. 4) of bacterial members in the ileum of two groups. Shigella was found to be enriched in the control, while the microbiota in EO-supplemented group was differentially enriched with Burkholderiales, Actinobacteria, Bifidobacteriales, Enterococccaceae, Bacillaceae, Kocuria and Corynebacteria. Compared with the control, dietary inclusion of EO increased (P < 0.05) the abundances of Cyanobacteria, Burkholderiales and Comamonadaceae and simultaneously tended to increase (P < 0.1) Actinobacteria.

Table 3 Effects of dietary supplementation with essential oil on laying performance of laying hens

| Items                        | Treatments^2 | SEM^3 | P-value |
|------------------------------|--------------|-------|---------|
|                              | Control      | EO100 | EO200   | EO400     |
| Egg production               |              |       |         |           |
| weeks 1–4                    | 0.880        | 0.905 | 0.907   | 0.905     | 0.0055  | 0.243  | 0.174  | 0.138  |
| weeks 5–8                    | 0.863        | 0.898 | 0.894   | 0.893     | 0.0064  | 0.183  | 0.197  | 0.134  |
| weeks 9–12                   | 0.854        | 0.861 | 0.885   | 0.861     | 0.0060  | 0.282  | 0.598  | 0.233  |
| weeks 1–12                   | 0.865        | 0.888 | 0.896   | 0.888     | 0.0050  | 0.144  | 0.163  | 0.064  |
| Average egg weight, g        |              |       |         |           |
| weeks 1–4                    | 60.51        | 60.27 | 61.13   | 61.85     | 0.27    | 0.147  | 0.031  | 0.092  |
| weeks 5–8                    | 60.65        | 60.24 | 61.31   | 62.03     | 0.26    | 0.079  | 0.017  | 0.052  |
| weeks 9–12                   | 60.47        | 60.08 | 60.99   | 61.36     | 0.24    | 0.240  | 0.083  | 0.216  |
| weeks 1–12                   | 60.55        | 60.19 | 61.14   | 61.75     | 0.24    | 0.110  | 0.028  | 0.083  |
| Average daily feed intake, g/hen per day |              |       |         |           |
| weeks 1–4                    | 111.66       | 111.28| 108.77  | 111.97    | 0.68    | 0.331  | 0.948  | 0.304  |
| weeks 5–8                    | 109.47       | 110.97| 108.64  | 111.02    | 0.66    | 0.524  | 0.592  | 0.768  |
| weeks 9–12                   | 108.30       | 109.72| 110.57  | 110.74    | 0.72    | 0.631  | 0.244  | 0.415  |
| weeks 1–12                   | 110.33       | 110.26| 108.68  | 111.08    | 0.54    | 0.467  | 0.683  | 0.411  |
| Feed conversion ratio, g/g    |              |       |         |           |
| weeks 1–4                    | 2.10         | 2.05  | 1.96    | 2.00      | 0.021   | 0.075  | 0.064  | 0.039  |
| weeks 5–8                    | 2.10         | 2.06  | 1.99    | 2.01      | 0.018   | 0.236  | 0.053  | 0.050  |
| weeks 9–12                   | 2.12^ab      | 2.10^a| 2.01^b  | 2.09^ab   | 0.015   | 0.029  | 0.272  | 0.048  |
| weeks 1–12                   | 2.11^a       | 2.07^ab| 1.98^b  | 2.03^ab   | 0.016   | 0.026  | 0.053  | 0.017  |

^1 n = 8 replicates per treatment
^2 Control, hens received a basal diet; EO100, EO200 and EO400, hens received a basal diet supplemented with 100, 200 or 400 mg/kg oregano essential oil, respectively
^3 SEM, standard error of the mean
a-b Values within a row with no common superscripts differ significantly (P < 0.05)
Table 4 Effects of dietary supplementation with essential oil on egg quality of laying hens1

| Items                        | Treatments2                  | SEM3 | P-value              |
|------------------------------|------------------------------|------|----------------------|
|                              | Control | EO100 | EO200 | EO400 | ANOVA | Linear | Quadratic |
| Eggshell thickness, 10-2 mm  |         |       |       |       |       |        |           |
| week 4                       | 42.31b  | 44.19a| 44.28a| 44.69a| 0.23  | 0.002  | 0.002     | 0.001     |
| week 8                       | 42.86b  | 44.21a| 44.39a| 44.54a| 0.18  | 0.001  | 0.002     | < 0.001   |
| week 12                      | 42.25b  | 44.00a| 44.10a| 44.56a| 0.20  | < 0.001| < 0.001   | < 0.001   |
| Eggshell strength, N         |         |       |       |       |       |        |           |
| week 4                       | 35.51   | 37.02 | 36.95 | 36.72 | 0.32  | 0.322  | 0.314     | 0.217     |
| week 8                       | 34.24   | 35.63 | 35.10 | 34.58 | 0.38  | 0.804  | 0.980     | 0.536     |
| week 12                      | 34.00   | 34.89 | 35.65 | 35.84 | 0.39  | 0.330  | 0.089     | 0.176     |
| Relative eggshell weight, %  |         |       |       |       |       |        |           |
| week 4                       | 9.43    | 9.67  | 9.45  | 9.55  | 0.058 | 0.430  | 0.795     | 0.915     |
| week 8                       | 9.26    | 9.52  | 9.44  | 9.57  | 0.051 | 0.135  | 0.065     | 0.136     |
| week 12                      | 9.26    | 9.68  | 9.55  | 9.62  | 0.063 | 0.075  | 0.125     | 0.107     |
| Shape index                  |         |       |       |       |       |        |           |
| week 4                       | 1.35    | 1.34  | 1.35  | 1.35  | 0.0039| 0.950  | 0.968     | 0.886     |
| week 8                       | 1.35    | 1.34  | 1.35  | 1.34  | 0.0031| 0.541  | 0.424     | 0.546     |
| week 12                      | 1.37    | 1.36  | 1.37  | 1.36  | 0.0026| 0.374  | 0.106     | 0.261     |
| Albumen height, mm           |         |       |       |       |       |        |           |
| week 4                       | 6.10    | 6.32  | 6.44  | 6.37  | 0.11  | 0.744  | 0.440     | 0.533     |
| week 8                       | 6.46    | 6.87  | 6.94  | 6.74  | 0.081 | 0.275  | 0.400     | 0.185     |
| week 12                      | 7.07    | 7.30  | 7.31  | 7.27  | 0.080 | 0.716  | 0.504     | 0.532     |
| Haugh unit                   |         |       |       |       |       |        |           |
| week 4                       | 75.88   | 77.51 | 77.93 | 77.36 | 0.89  | 0.873  | 0.628     | 0.705     |
| week 8                       | 79.08   | 82.21 | 81.38 | 80.32 | 0.59  | 0.266  | 0.772     | 0.231     |
| week 12                      | 82.93   | 84.74 | 82.17 | 83.94 | 0.67  | 0.564  | 0.859     | 0.959     |
| Yolk color                   |         |       |       |       |       |        |           |
| week 4                       | 5.65    | 5.83  | 5.60  | 5.90  | 0.11  | 0.735  | 0.512     | 0.760     |
| week 8                       | 5.45    | 5.80  | 5.63  | 5.93  | 0.080 | 0.167  | 0.071     | 0.197     |
| week 12                      | 4.83    | 4.90  | 4.78  | 4.85  | 0.064 | 0.927  | 0.974     | 0.990     |

1 n = 8 replicates per treatment
2 Control, hens received a basal diet; EO100, EO200 and EO400, hens received a basal diet supplemented with 100, 200 or 400 mg/kg oregano essential oil, respectively
3 SEM, standard error of the mean
4 Values within a row with no common superscripts differ significantly (P < 0.05)

Table 5 Effects of dietary supplementation with essential oil on ileal morphology of laying hens

| Items       | Treatments3                  | SEM4 | P-value              |
|-------------|------------------------------|------|----------------------|
|             | Control | EO100 | EO200 | EO400 | ANOVA | Linear | Quadratic |
| VH, μm      | 587.61  | 609.70| 731.26 | 585.89 | 25.92  | 0.145  | 0.922     | 0.153     |
| CD, μm      | 149.58  | 144.35| 161.19 | 125.41 | 5.67   | 0.156  | 0.159     | 0.143     |
| VCR         | 4.02    | 4.23  | 4.60  | 4.64  | 0.11   | 0.128  | 0.029     | 0.064     |

4 n = 8 replicates per treatment at the end of week 12
5 VH, villus height; CD, crypt depth; VCR, villus height to crypt depth ratio
6 Control, hens received a basal diet; EO100, EO200 and EO400, hens received a basal diet supplemented with 100, 200 or 400 mg/kg oregano essential oil, respectively
7 SEM, standard error of the mean
(Actinobacteria) and Bifidobacteriales (Bifidobacteriaceae) abundances (Table 7).

**Correlation between ileal microbiota and mucosal gene expression, production performance, digestive enzyme activity or gut morphology**

A Pearson correlation analysis was employed to determine whether there was any association among laying performance, intestinal morphology, digestive enzyme activity, mucosal gene expression and main bacterial members. Correlation analysis revealed that the mRNA expression of IL-1β and TNF-α was negatively correlated \((P < 0.05)\) with the abundances of Bacillaceae, whereas TNF-α and IFN-γ expression were positively correlated \((P < 0.05)\) with Moraxellaceae and Clostridiaceae abundances, respectively. VCR was negatively correlated \((P < 0.05)\) with Moraxellaceae, but it had a positive correlation \((P < 0.05)\) with Corynebacteriaceae. FCR showed a positive correlation \((P < 0.05)\) with Lactobacillaceae, while showed a negative correlation \((P < 0.05)\) with Comamonadaceae and Oxalobacteraceae. There was a positive correlation \((P < 0.05)\) between the activity of chymotrypsin and the abundances of Comamonadaceae, Bifidobacteriaceae, Oxalobacteraceae, Enterococcaceae and Caulobacteraceae, and a negative correlation \((P < 0.05)\) between the activity of lipase and Comamonadaceae, Bifidobacteriaceae and Caulobacteraceae abundances. At genus level, the mRNA expression of ZO-1 was positively correlated \((P < 0.05)\); Fig. 5b) with Silene abundance, but IL-1β expression was negatively correlated \((P < 0.05)\) with the number of Bacillaceae_Bacillus. The abundance of Acinetobacter was positively correlated \((P < 0.05)\) with TNF-α expression, but was negatively correlated \((P < 0.05)\) with VCR. FCR showed a positive correlation \((P < 0.05)\) with the abundances of Aquabacterium, Ralstonia and Pelomonas, but showed a positive correlation \((P < 0.05)\) with Lactobacillus abundance. The abundances of Aeriscardovia, Aquabacterium, Pelomonas and Caulobacter were positively correlated \((P < 0.05)\) with lipase and chymotrypsin activity, and chymotrypsin activity was positively correlated \((P < 0.05)\) with Ralstonia and Silene abundances.

**Discussion**

In the present study, dietary EO supplementation improved feed efficiency but showed no statistical effects on egg production of late-phase hens. Consistent with our findings, several recent studies have indicated that EO improved the feed utilization in broilers [21, 22] and laying hens [18, 23]. These beneficial effects could be attributed to the active components (thymol and carvacrol) in EO, which have been demonstrated to exhibit antimicrobial, anti-inflammation activities as well as improvements in gut health status and utilization of nutrients [4, 24]. It may be of great importance to alleviate...
age-related deleterious effects on intestinal health and functions, since intestinal health problems were regarded as a crucial reason for poor laying performance in late-phase hens [3, 25]. In this study, the increased VCR and digestive enzyme activities, improved immune homeostasis and altered microbiota structure suggested an enhancement of digestion and absorption function and a healthy condition of intestine, thus benefiting feed utilization of laying hens. In contrast, other studies suggested that EO or their main compounds yielded no significant improvements in terms of laying performance of laying hens [26, 27]. The inconsistencies in the efficacy of EO on production performance may be related to the composition and supplemental levels of EO, the basal diet, bird age, and the environmental conditions. Poor physiological conditions of late-phase laying hens in this study may favor the efficacy of EO on feed utilization. It was reported that carvacrol or thymol at high concentrations might exert negative effects on intestinal epithelial cells and gut beneficial bacteria [28, 29], which may subsequently compromise feed utilization of laying hens. It was speculated that dietary EO supplementation exhibited favorable effects on feed utilization at an appropriate level (200 mg/kg), whereas these improvements may be masked by adverse effects at a higher concentration (400 mg/kg). However, the double-edged effects of EO on intestinal functions and gut microbiota need further investigation. In this study, the medium dose (200 mg/kg) was the most effective in terms of production performance and egg quality of laying hens.

It is well known that digestive enzymes, including chymotrypsin, α-amylase and lipase, are involved in nutrient digestion for absorption and their activities, which are crucial for feed utilization and production performance of animals. It has been reported that EO could stimulate digestive secretions such as bile acids, gastric and digestive enzymes (e.g., lipase, amylase and proteases) in rats [30]. Similarly, the use of phytopharmacological products containing thymol, carvacrol or other active components in the diets of broilers and pigs elevated the activities of intestinal amylase, protease, and lipase [10]. In this study, there was an improvement in the activities of ileal chymotrypsin and lipase in response to the increased EO addition. It could be postulated that dietary EO supplementation could enhance the digestive

Fig. 2 Alpha (a) and Beta (b) diversity analysis of ileal microbiota from laying hens. Beta diversity analysis with principal coordinates analysis (PCoA) was based on the unweighted UniFrac distance. c, control; e, essential oil-supplemented group (control + essential oil addition at 200 mg/kg)
enzyme activities, possibly resulting in the accelerated digestion of protein and fat and thus assisting with their absorption in the intestine. The increased digestive enzyme activities might be due to the antibacterial activity of thymol and carvacrol, and their modulatory effects on intestinal microbial composition [4], resulting in less pathogen-induced damage of enterocytes. It would further diminish the risk of pathogen invasion to intestinal epithelial cells and promote their ability to regenerate villus. Mature villus cells implied they are more active to secret enzymes than immature crypt cells [31]. This was supported by the findings that the addition of EO, with thymol or carvacrol as active compounds, exhibited positive effects on intestinal morphology, evidenced by increased VH and VCR accompanied with decreased CD [6, 13, 17]. Likewise, in this study, there was a linear increase in VCR with the increased EO addition, indicating an enhancement of absorptive surface area, efficient enzyme secretion and nutrient transport, possibly benefiting nutrient utilization in the intestine. The declined absorption efficiency of intestinal calcium has been recognized as the main cause for the poor eggshell quality in late-phase laying hens [32, 33]. In this study, the enhanced villi-crypts absorptive area following EO addition could be favorable to calcium absorption [34], resulting in an elevation in calcium deposition into eggshell along with increased eggshell thickness. Another potential mechanism reported was that EO could improve uterine health and an appropriate site would be provided for eggshell calcification, consequently increasing eggshell weight and thickness [18]. Thus, the improvements of eggshell quality in response to EO supplementation may be attributed to the beneficial effects of dietary EO addition on intestinal and uterine health, whereas the underlying mechanisms need to be further studied.

Disruption of tight junctions and microbiota dysbiosis due to long-term egg production would enable the translocation of luminal pathogens and toxins [35, 36]. It would subsequently lead to inflammation and tissue damage, which may be partially responsible for the lower nutrient absorption and the compromised laying performance of laying hens in the late production period. In this study, EO addition down-regulated mRNA expression of TLR-4 and pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ, while the expression of anti-inflammatory cytokines in the ileum was not affected, which were consistent with previous studies in broilers [13, 22]. TLRs, the core components in mucosal innate

![Fig. 3 Relative abundance of ileal microbiota from laying hens.](image_url)
immune responses, can recognize microbiota and their products and finally initiate inflammatory responses with the release of pro-inflammatory cytokines [37]. TLR-4 is involved in the recognition of lipopolysaccharide (LPS), a unique component of the outer membrane of gram-negative bacteria such as *Escherichia coli*, *Salmonella* and *Shigella* strains. The decreased expression of TLR-4, IL-1β, TNF-α and IFN-γ might be associated with the antimicrobial properties of EO, suggesting that EO could exert anti-inflammatory activity in the ileum of laying hens, possibly by suppressing the activation of TLR4-mediated signaling pathway. In fact, anti-inflammatory activities of thymol and carvacrol have been well documented. They can suppress the expression of proinflammatory cytokines, stimulate the expression of anti-inflammatory cytokines, prevent inflammatory cell recruitment and thus attenuate inflammation [38, 39]. However, in this study, the expression of anti-inflammatory cytokines was not affected by EO treatment and the exact mechanism of anti-inflammatory activity of EO in laying hens required further investigation. Additionally, TLRs are also involved in the

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**Fig. 4** Linear discriminant analysis (LDA) combined effect size measurements (LEfSe) analysis of ileal microbiota. C, control; E, essential oil-supplemented group (essential oil addition at 200 mg/kg). Species with significant difference that have an LDA score greater than the estimated value (3.0). The length of the histogram represents the LDA score.
regulation of intestinal barrier integrity [40]. Pro-inflammatory cytokines, such as IL-1β, TNF-α and IFN-γ, can activate NF-κB signaling pathway and then impair the epithelial barrier function by dysregulating tight junctions [41, 42]. ZO-1, one of the tight junction proteins, is an essential component of intestinal barrier and plays a crucial role in regulating intestinal permeability and integrity [43]. In the present study, oregano EO supplementation upregulated the mRNA expression of intestinal barrier gene ZO-1 in the ileum compared to that in the control, which may be associated with the suppressed expression of pro-inflammatory cytokines. It indicated that oregano EO could improve the barrier function of intestinal epithelium and consequently strengthen immune defense against pathogen infection. Similarly, dietary inclusion of thymol or carvacrol was reported to upregulate the mRNA expression of occludin, ZO-1 and claudin-1 in small intestine of broiler chickens regardless of C. perfingens challenge [6, 13]. Thymol treatment could enhance the barrier function of epithelial cells by increasing the protein level of ZO-1 in the IPEC-J2 cell model [44]. Therefore, the improved immune status and strengthened epithelial barrier in response to EO treatment would be beneficial for the maintenance of gut health and production performance of laying hens.

To better understand the favorable effects of EO, further analysis was conducted on gut microbiota, whose interactions with gut play a crucial role in prevention of pathogen colonization, maintenance of immune homeostasis and metabolism of nutrient. Since the favorable effects of EO supplementation were mainly observed in EO200 group, the modulatory roles of EO on intestinal microbial composition were assessed in the control and EO200 groups. Herein, there was no difference in the alpha-diversity of the intestinal microbiota between

Table 7 Differences of bacterial distribution in ileal digesta between the control and essential oil supplementation groups

| Items, % | C | E | P-value |
|---------|---|---|---------|
| Phyla   |   |   |         |
| Actinobacteria | 1.48 ± 2.86 | 10.68 ± 8.88 | 0.052 |
| Cyanobacteria | 0.63 ± 0.81 | 9.13 ± 7.66 | 0.042 |
| Classes   |   |   |         |
| Betaproteobacteria | 0.74 ± 0.79 | 12.77 ± 8.71 | 0.019 |
| Actinobacteria | 1.48 ± 2.85 | 10.59 ± 8.83 | 0.053 |
| Chloroplast | 0.63 ± 0.81 | 9.12 ± 7.66 | 0.042 |
| Alphaproteobacteria | 0.32 ± 0.39 | 2.53 ± 1.12 | 0.004 |
| Orders    |   |   |         |
| Burkholderiales | 0.73 ± 0.79 | 12.76 ± 8.70 | 0.019 |
| Bifidobacteriales | 1.43 ± 2.80 | 10.04 ± 8.68 | 0.060 |
| Streptophyta | 0.63 ± 0.81 | 9.12 ± 7.66 | 0.042 |
| Families   |   |   |         |
| Comamonadaceae | 0.60 ± 0.66 | 10.97 ± 7.42 | 0.019 |
| Bifidobacteriaceae | 1.43 ± 2.80 | 10.04 ± 8.68 | 0.060 |
| Oxalobacteraceae | 0.12 ± 0.12 | 1.77 ± 1.33 | 0.013 |
| Genera     |   |   |         |
| Aeriscardovia | 1.41 ± 2.80 | 9.86 ± 8.69 | 0.064 |
| Aquabacterium | 0.40 ± 0.54 | 8.89 ± 6.00 | 0.018 |
| Ralstonia | 0.10 ± 0.10 | 1.69 ± 1.34 | 0.033 |
| Pelomonas | 0.07 ± 0.05 | 1.69 ± 1.46 | 0.023 |
| SMB53 | 1.56 ± 1.61 | 0.18 ± 0.28 | 0.090 |
| Silene | 0.23 ± 0.30 | 1.39 ± 1.06 | 0.027 |

| a Data are represented with the means ± standard deviation (n = 6) |
|                   b C, control; E, essential oil-supplemented group (essential oil addition at 200 mg/kg) |

Fig. 5 Pearson’s correlation analysis between the abundances of ileal microbiota and production or intestinal parameters. a at family level and b at genus level. The intensity of the colors represents the degree of association. Red represents a significant positive correlation (P < 0.05), blue represents significantly negative correlation (P < 0.05), and white shows that the correlation was not significant (P > 0.05)
groups, whereas the results of beta-diversity analysis showed significant clustering according to dietary treatments, indicating that ileal microbiota community structure was altered by EO addition. Then, further analysis was performed on alteration of microbiota composition and specific taxa following EO addition. The enhanced digestive enzyme activity of ileum digesta in this study might be associated with the modulatory effects of EO on intestinal microbial composition. Actinobacteria, Bifidobacteriales, Deinococcus, Bacillaceae and Caulobacterales have been reported to be related to the improvement of animal feed utilization through producing extracellular enzymes (e.g., amylases and proteases) [45, 46]. Furthermore, Actinobacteria and Caulobacteria were characterized with their significant capacity of decomposing undigested components in feeds by secreting endogenous enzymes (cellulases, chitinases, xylanases, and pectinase) [47]. These enzymes can partially hydrolyze low-digestible components in poultry diets and mitigate the antinutritional effects by reducing the viscosity of gut digesta, thus improving nutrient digestion and absorption [5]. It was supported by the findings in this study that Bifidobacteriaceae (Aeriscardovia) and Caulobacteriaceae (Caulobacter) were positively correlated with the activity of chymotrypsin and lipase. Therefore, the enrichment of Actinobacteria, Bifidobacteriales, Deinococcus, Bacillaceae and Caulobacterales in EO supplementation group could represent the promotion of endogenous digestive enzymes secretion, possibly favoring the increased activity of digestive enzymes and the improved feed utilization efficiency.

The antimicrobial activity of EO containing carvacrol or thymol has been widely tested against poultry pathogens such as Escherichia-Shigella [48], Campylobacter [49] and Salmonella spp. [50]. The antimicrobial mechanisms may be related to the perturbation of the lipid fraction in the plasma membrane, resulting in the altered membrane permeability and the leakage of intracellular materials of pathogen bacteria [51, 52]. In this study, dietary supplementation with EO inhibited the number of pathogens (Shigella) in the ileum compared to those in the control. Shigella was the core component of bacterial diarrhea in human and animals [53], and its invasion can cause physical impairment of tight junctions through direct interactions between secreted bacterial products and intestinal epithelial cells [54]. Numerous studies showed that the increased abundance of Shigella was closely associated with the poor animal production performance, the destroyed intestinal integrity and the raised levels of pro-inflammatory cytokines [55]. In addition, EO treatment could increase the abundances of some beneficial bacteria such as Actinobacteria, Bifidobacteriales, Enterococcaceae and Bacillaceae, which are helpful for the maintenance of overall microbial structure. Actinobacteria was regarded as keystone taxa to modulate the functionality of intestinal microbiota owing to the production of bacteriocins [46] and their ability to convert feedstuff into fermentable microbial biomass [56]. Enterococcus, Bifidobacteria and Bacillus have attracted great interests as natural antimicrobial probiotics to prevent diarrhea, improve feed efficiency and promote growth in animal production [57, 58]. They are capable to protect against potential pathogens by producing bacteriocins and blocking the adhesion of pathogens to the intestinal mucosa [59–61]. Thus, these results indicate that dietary EO inclusion may be beneficial for inhibiting pathogen colonization in the ileum of laying hens. In addition, Bifidobacteria, as the dominant microflora in normal intestine [62], could reinforce intestinal mucosal immune barrier functions by increasing the number of goblet cells and the secretion of mucin-2 [63]. Members of Bacillus are reported to participate in initiating and regulating immune responses by regulating cytokine expression [64] and showed positive association with the enhancement of intestinal tight junctions [46, 65]. Similarly, this study indicated that Bacillaceae abundance had a negative relationship with the expression of ileal pro-inflammatory cytokines (IL-1β and IFN-γ). Corynebacteriaceae may exert modulatory effects on inflammation responses, whose members like Corynebacterium pyruvicproduces, have been shown to stimulate the maturation and proliferation of dendritic cells and up-regulate Th2 responses in mice [66]. The functions of Burkholderia and Kocuria in inflammation responses were still unknown. However, an increase in gut Burkholderia abundance was accompanied by the improvements of intestinal structure and growth performance in chickens following a probiotic addition [67], and accompanied by the attenuation of plant extracts on S. pullorum-induced gut inflammation and the improvements of laying performance [68], implying a favorable effect of Burkholderia on gut health of chickens. Decreased population of Kocuria was one of the specific changes responsible for gut microbiota dysbiosis caused by Salmonella enterica serovar Infantis [69]. Conversely, the expansion of Kocuria was postulated to be responsible for decreased levels of proinflammatory cytokines IL-6, 8 and IFN-γ in neonatal piglets treated with sodium butyrate [70], indicating its contribution to alleviating intestinal inflammation. In this study, the suppressed abundance of pathogens as well as the increased abundance of some health-promoting bacteria might conduce to the improved gut morphology, enhanced epithelial barrier functions and the amelioration of intestinal inflammation in EO supplemented group (Fig. 6).

Conclusions
In conclusion, this study demonstrated that dietary EO supplementation decreased feed efficiency and enhanced eggshell quality of late-phase laying hens by improving
gut morphology, digestive enzyme activity, epithelial barrier functions and immune status, which could be in part responsible by the modulation of gut microbial profile. These findings may provide insights into the underlying mechanism of regulatory roles of EO on production performance and gut health in the late phase of production of laying hens.

Abbreviations
CD: Crypt depth; EO: Essential oil; FCR: Feed conversion ratio; IFN-γ: Interferon-γ; IL: Interleukin; LDA: Linear discriminant analysis; LEfSe: Linear discriminant analysis combined effect size measurements; PCoA: Principal coordinate analysis; TLR: Toll-like receptors; TNF-α: Tumor necrosis factor-α; VCR: Villus height to crypt depth ratio; VH: Villus height; ZO-1: Zonula occludens-1

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Authors’ contributions
JF and MYL conducted the animal feeding trial and performed the sample analyses; JF analyzed the data and wrote the manuscript; JW, SGW, HJZ and KQ assisted with data analysis; JW and GHQ contributed to the experimental design and the revision of manuscript. All authors have read and approved the manuscript.

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Availability of data and materials
The sequencing datasets are available in the Sequence Read Archive of National Center for Biotechnology Information (accession number: PRJNA693086).

Declarations

Ethics approval and consent to participate
The animal protocol was approved by the Animal Care and Use Committee of the Feed Research Institute of Chinese Academy of Agricultural Sciences.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Bain MM, Nys Y, Dunn IC. Increasing persistency in lay and stabilising egg quality in longer laying cycles. What are the challenges? Br Poult Sci. 2016;57(3):330–8. https://doi.org/10.1080/00071668.2016.1161727.
2. Rattanawut J, Pimpka O, Yamauchi KE. Effects of dietary bamboo vinegar supplementation on performance, eggshell quality, ileal microflora composition, and intestinal villus morphology of laying hens in the late phase of production. Anim Sci J. 2018;89(11):1572–80. https://doi.org/10.1111/asj.13080.
3. Wang WW, Wang J, Zhang HJ, Wu SG, Qi GH. Effects of Clostridium butyricum on production performance and intestinal absorption function of laying hens in the late phase of production. Anim Feed Sci Technol. 2020;264:114476. https://doi.org/10.1016/j.anifeedsci.2020.114476.
4. Zeng Z, Zhang S, Wang H, Piao X. Essential oil and aromatic plants as feed additives in non-ruminant nutrition: a review. J Anim Sci Biotechnol. 2015;6(1):7. https://doi.org/10.1186/s40104-015-0004-5.
5. Hashemipour H, Khaksar V, Rubio LA, Veldkamp T, Van Krimpen MM. Effect of feed supplementation with a thymol plus carvacrol mixture, in combination or not with an NSP-degrading enzyme, on productive and physiological parameters of broilers fed on wheat-based diets. Anim Feed Sci Technol. 2016;211:117–31. https://doi.org/10.1016/j.anifeedsci.2015.09.023.
6. Pham VH, Kan L, Huang J, Geng Y, Zhen W, Guo Y, et al. Dietary encapsulated essential oils and organic acids mixture improves gut health in broiler chickens challenged with necrotic enteritis. J Anim Sci Biotechnol. 2020;11(1):18. https://doi.org/10.1186/s40104-019-0421-y.
21. Reyer H, Zentek J, Männer K, Youssef I, Aumiller T, Weghuber J, et al.
20. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, et al.
19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using
18. Abdel-Wareth AAA, Lohakare JD. Productive performance, egg quality,
17. Du E, Wang W, Gan L, Li Z, Guo S, Guo Y. Effects of thymol and carvacrol
16. Awaad MHH, Elmenawey M, Ahmed KA. Effect of a specific combination of
15. Yang C, Kennes YM, Lepp D, Yin X, Wang Q, Yu H, et al. Effects of supplemental thymol
14. Yin D, Du E, Yuan J, Gao J, Wang Y, Aggrey SE, et al. Supplemental thymol and carvacrol
13. Du E, Wang W, Gan L, Li Z, Guo S, Guo Y. Effects of thymol and carvacrol
12. Awaad MHH, Elmenawey M, Ahmed KA. Effect of a specific combination of
11. Lee SH, Lillehøi HS, Jiang SJ, Lee KW, Bravo D, Lillehøi EP. Effects of dietary supplementation with phytonutrients on vaccine-stimulated immunity against infection with Eimeria tenella. Vet Parasitol. 2011;181(2-4):97–105. https://doi.org/10.1016/j.vetpar.2011.05.003.
10. Awaad MHH, Elmenawey M, Ahmed KA. Effect of a specific combination of carvacrol, cinnamaldehyde, and on the growth performance, carcass quality and gut integrity of broiler chickens. Vet World. 2014;7(5):284–90. https://doi.org/10.14202/vetworld.2014.7.5.0284.
9. Jang IS, Ko YH, Kang SY, Lee CY. Effect of a commercial essential oil on growth performance, nutrient digestibility and yolk fatty acid profile in laying hens. Anim Nutr. 2017;3(2):127–31. https://doi.org/10.1016/j.aninut.2017.03.005.
8. Cheng C, Xia M, Zhang X, Wang C, Jiang S, Peng J. Supplementing oregano essential oil in a reduced-protein diet improves growth performance and nutrient digestibility by modulating intestinal bacteria, intestinal morphology, and antioxidative capacity of growing-finishing pigs. Animals. 2018;8(9):159. https://doi.org/10.3390/ani8090159.
7. Zou Y, Xiang Q, Wang J, Peng J, Wei H. Oregano essential oil improves intestinal morphology and expression of tight junction proteins associated with modulation of selected intestinal bacteria and immune status in a pig model. Biomed Res Int. 2016;2016:5436738.
6. Zou Y, Xiang Q, Wang J, Peng J, Wei H. Oregano essential oil improves intestinal morphology and expression of tight junction proteins associated with modulation of selected intestinal bacteria and immune status in a pig model. Biomed Res Int. 2016;2016:5436738.
5. Reyer H, Zentek J, Männer K, Youssef I, Aumiller T, Weghuber J, et al.
4. Cheng C, Xia M, Zhang X, Wang C, Jiang S, Peng J. Supplementing oregano essential oil in a reduced-protein diet improves growth performance and nutrient digestibility by modulating intestinal bacteria, intestinal morphology, and antioxidative capacity of growing-finishing pigs. Animals. 2018;8(9):159. https://doi.org/10.3390/ani8090159.
3. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using
2. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, et al.
1. Feng et al. Journal of Animal Science and Biotechnology (2021) 12:72 Page 14 of 15
light-chain kinase pathways. Biochem Biophys Res Commun. 2012;428(2):321–6. https://doi.org/10.1016/j.bbrc.2012.10.056.

42. Wang H, Zhao JX, Hu N, Ren J, Ju M, Zhu MJ. Side-stream smoking reduces intestinal inflammation and increases expression of tight junction proteins. World J Gastroenterol. 2012;18(28):3867–70. https://doi.org/10.3748/wjg.v18.i28.3867.

43. Gianni S, Howarth GZ, Nathrus G, Kitesma SM, Barakatian R, Forder R, et al. Gene expression and morphological changes in the intestinal mucosa associated with increased permeability induced by short-term fasting in chickens. J Anim Physiol Anim Nutr. 2018;102(2):663–61. https://doi.org/10.1111/jpn.13088.

44. Omonjoo FA, Liu S, Hui Q, Zhang H, Lahaye L, Bodin JC, et al. Thymol improves barrier function and attenuates inflammatory responses in porcine intestinal epithelial cells during lipopolysaccharide (LPS)-induced inflammation. J Agric Food Chem. 2019;67(2):615–24. https://doi.org/10.1021/acs.jafc.8b05480.

45. Li IC, Wu SY, Liou JC, Liu HH, Chen CH, Chen CC. Effects of Deinococcus spp. supplement on egg quality traits in laying hens. Poult Sci. 2018;97(1):319–27. https://doi.org/10.3382/ps.pex281.

46. Ma Y, Wang W, Zhang H, Wang J, Zhang W, Gao J, et al. Supplemental Bacillus subtilis DSM 32315 manipulates intestinal structure and microbial composition in broiler chickens. Sci Rep. 2018;8(1):15358. https://doi.org/10.1038/s41598-018-33762-8.

47. Pinnell LJ, Dunford E, Ronan P, Hausner M, Neufeld JD. Recovering microbiota and systemic immunity. J Anim Sci Biotechnol. 2020;11:101. https://doi.org/10.1186/s40104-015-0055-7.

48. Elshaghabee F, Rokana N, Gulhane RD, Sharma C, Panwar H. Bacillus as potential probiotics: status, concerns, and future perspectives. Front Microbiol. 2019;10:15058. https://doi.org/10.3389/fmicb.2019.015058.

49. Alali WQ, Hoface CL, Mathis GF, Falys G. Effect of essential oil compound on shedding and colonization of Salmonella enterica serovar Heidelberg in broilers. Poult Sci. 2013;92(2):836–41. https://doi.org/10.3382/ps.p2012-07283.

50. Du E, Gan L, Li Z, Wang W, Liu D, Guo Y. In vitro antibacterial activity of thymol and carvacrol and their effects on broiler chickens challenged with Clostridium perfringens. J Anim Sci Biotechnol. 2015;6(1). https://doi.org/10.1186/s40104-015-0055-7.

51. Stevanović ZD, Božnjak-Neumüller J, Pajić-Ljakić I, Raj J, Vasiljević M. Essential oils as feed additives-future perspectives. Molecules. 2018;23(7):1717. https://doi.org/10.3390/molecules23071717.

52. Fischer Walker CL, Sack D, Black RE. Etiology of diarrhoea in older children, adolescents and adults: a systematic review. Plos Neglect Trop Dis. 2010;4(8):e768. https://doi.org/10.1371/journal.pntd.0000768.

53. Sakaguchi T, Köhler H, Gu X, McCormick BA, Reinecker HC. Shigella. Gut 2012;61:1261. 54. Gilani S, Howarth GZ, Nathrus G, Kitesma SM, Barakatian R, Forder R, et al. Gene expression and morphological changes in the intestinal mucosa associated with increased permeability induced by short-term fasting in chickens. J Anim Physiol Anim Nutr. 2018;102(2):663–61. https://doi.org/10.1111/jpn.13088.

55. He Y, Jinno C, Kim K, Wu Z, Tan B, Li X, et al. Dietary Bacillus spp enhanced growth and disease resistance of weaned pigs by modulating intestinal microbiota and systemic immunity. J Anim Sci Biotechnol. 2020;11:101.