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Effect of Supplemental Oregano Essential Oils in Diets on Production Performance and Relatively Intestinal Parameters of Laying Hens

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Abstract

This study was designed to investigate the effects of dietary oregano essential oils on 150 30-week-old Hy-Line Layers' productive performance, egg quality characteristics, cecal microbiota, digestive enzyme activities, mucosa structure of the duodenum and jejunum and glucose and small peptides transporters expression in the duodenum and jejunum. All hens were allocated into one of five different groups: negative control (NC; basal diet only), antibiotics control (CS; basal diet plus 100 mg/kg of 10% colistin sulfate) and treatments I, II, and III (basal diet supplemented with 50, 100 and 150 mg/kg oregano essential oils, respectively). The results showed that the laying rate, average egg weight, feed conversion ratio and the activities of amylase and trypsin were significantly improved by a diet supplemented with 100 mg/kg oregano essential oils ($P < 0.01$). The addition of oregano essential oils increased the number of intestinal *Bifidobacterium* and *Lactobacillus* significantly ($P < 0.01$), whereas the number of intestinal *Escherichia coli* and *Salmonella* was significantly ($P < 0.01$) decreased. The addition of 100 mg/kg oregano essential oils increased duodenum villus height ($P > 0.05$), significantly increased duodenum villus-height-to-crypt-depth ratios ($P < 0.01$) and decreased crypt depth in the duodenum ($P < 0.05$). Furthermore, the glucose transporter 2 (GLUT2), peptide transporter 1 (PepT1) and sodium-glucose cotransporter 1 (SGLT1) gene expression levels in the duodenum and jejunum were significantly increased in laying hens on a diet supplemented with 100 mg/kg oregano essential oils ($P < 0.01$). However, egg weight, relative eggshell weight, yolk index and Haugh unit value were not significantly affected by the addition of oregano essential oils ($P > 0.05$).

Keywords

Laying Hens, Oregano Essential Oils, Intestinal Morphology, Intestinal Function

1. Introduction

It has been widely recognized that the presence of intestinal diseases, such as bacterial and viral infections, imbalances of the gut flora and coccidiosis, can affect nutrient utilization adversely and reduce the production performance of laying hens. To prevent intestinal diseases and improve production performance, a variety of feed additives, especially antibiotics, have been widely used in the poultry industry for several decades. The use of antibiotics in poultry feed has led to growing concern about drug residues and the development of antimicrobial resistance [1] [2]. Since the implementation of a complete ban on antibiotic feed additives by the European Union in 2006, finding suitable alternatives to antibiotics has become more important [3]. The alternatives most frequently used as feed additives for increasing performance and general health are prebiotics, probiotics, organic acids and phytogenic additives. Phytogenic additives, which are found in a wide variety of plants, spices and their derivatives, positively affect the quality of products, production performance and animal health and have been recognized as safe in the food industry [3].

Oregano (*Origanum vulgare* L.) essential oils, a phytogenic additive, are an aromatic plant that is indigenous to the Mediterranean region. It has been reported that oregano essential oils has many diverse biological activities *in vitro* and *in vivo*, including antimicrobial, antioxidant and antifungal effects, which mainly depended on the carvacrol and thymol compositions [4] [5] [6] [7]. Several *in vivo* studies on intestinal digestion have been conducted on the performance, cecal flora, digestive enzyme activities and intestinal morphology of poultry with oregano essential oils or combinations [8] [9] [10] [11], but the results have been variable and incomplete. Roofchaee found that OEO (oregano essential oil) exerted growth promoting effects and also displayed potent antibacterial effects against cecal *E. coli*. [8]. Hashemipour discovered that feed supplementation with thymol and carvacrol has a positive effect on broilers. It enhanced performance, increased antioxidant enzyme activities, retarded lipid oxidation, and enhanced digestive enzyme activities [9]. Radwan also found that oregano or thyme at 1.0% can improve productive performance [11]. The effects and mechanisms of oregano essential oils on intestinal digestive and absorption functions in laying hens have not been thoroughly investigated. Therefore, this study was conducted to investigate the effects of oregano essential oils as a laying hen feed supplement on the production performance, egg quality, cecal flora, morphological parameters, intestinal digestive enzyme activities and nutrition transport.

2. Materials and Methods

2.1. Animals, Housing and Diets

Experiments were conducted in accordance with the guiding principles in the use of animals adopted by the Chinese Association for Laboratory Animal Sciences. The study protocol was approved by the Ethics Committee on the Use and Care of Animals, Heilongjiang Bayi Agricultural University (Daqing, China). One hundred and fifty 30-week-old Hy-Line Layers were purchased from a local poultry farm, at the peak egg production period, and were housed alone in cages. The hens were randomly divided into five dietary treatments with thirty replicates. The hens were raised in a naturally

ventilated, open-side experimental house, and the environmental temperature of the room ranged from 20˚C to 27˚C. The relative humidity was between 45% and 60%, with 16 hours of light from 6 am to 10 pm, which followed the commercial recommendations.

The experiment lasted for 49 days after a 7-day adaptation period. The ingredient and nutrient levels of the basal diet present in Table 1 met the NRC recommendations (NRC, 1994). All hens were allocated into one of five different groups: negative control (NC; basal diet only), antibiotics control (CS; basal diet plus 100 mg/kg of 10% colistin sulfate) and treatments I, II, and III (basal diet supplemented with 50, 100 and 150 mg/kg oregano essential oils, respectively). The oregano essential oils components were identified by gas chromatography/mass spectrometry (GC/MS), and analysis by GC/MS with a chromatograph interfaced to a mass spectrometer (HP 5971, USA), present on Table 2. It is yellow fine granules which is packaged with microcapsule. The hens were quantitatively fed two times a day at the same time with free access to feed and water. All kinds of feed supplements used in the experiment were homogenously incorporated into the feed mixture in the feed mill.

Table 1. Ingredients and nutrient levels of the basal diet (air dry basis, %).

Items	Content
Ingredients	
Corn	61.52
Soybean meal	18.86
Cottonseed meal	6.29
Soybean oils	1.01
Limestone	8.43
NaCl	0.47
Lysine	0.03
DL-Methionine	0.40
Choline chloride (50%)	0.28
$CaHPO_4$	1.71
Premix[a]	1.00
Total	100.00
Nutrient levels	
Metabolic energy/(MJ/kg)	2.79
Crude protein	15.9
Total phosphorus	0.62
Available phosphorus	0.40
Methionine + Cysteine	0.63
Methionine	0.39
Lysine	0.71
Calcium	3.49

[a]The premix provides per kilogram of diet: Cu 5.00 mg; Fe 69.00 mg; Zn 84.00 mg; Mn 9816 mg; I 1.14 mg; Se 0.30 mg; VA 15,000 IU; VD 33,000 IU; VE 25.5 mg; VK 32.1 mg; VB1 2.4 mg; VB2 9 mg; VB6 5.1 mg; VB12 0.02 mg; calcium pantothenate 12 mg; niacin 48 mg; folic acid 1.2 mg; biotin 0.06 mg; roxarsone 50 mg; salinomycin 90 mg.

Table 2. Composition of essential oils of oregano obtained by GC/MS.

Constituent	Yield (%)
α-Pinene	0.32
β-Pinene	0.15
β-Myrcene	1.27
α-Thujene	0.79
Camphene	0.33
α-Terpinene	1.01
γ-Terpinene	1.17
D-Limonene	0.21
Terpinen	0.15
Methyl thymyl ether	0.72
Myristicacid	0.54
2-Isopropyholuene	1.38
2-Carene	0.59
Caryophyllene	3.70
Germacrene D	0.26
α-Farnesene	0.96
Sabinene	0.58
cis-*β*-Terpinene	0.49
Thymol	7.29
Terpinolene	0.17
δ-Cadinene	0.12
3-Octanol	0.23
p-Cymene	3.90
Carvacrol	73.06
Borneol	0.27

2.2. Productive Performance and Egg Quality

During the trial, egg production and egg weight (EW) were recorded daily by replicate, and egg conversion was calculated. Feed intake was recorded weekly, and egg mass, egg production, and feed conversion ratio (grams of feed consumed per grams of egg produced, FCR) were calculated. Deaths were recorded when they occurred.

On the last week of trial, 30 eggs were randomly collected from each replicate to determine the egg shape index, yolk index, yolk ratio, eggshell ratio and Haugh units [12]. Initially, the egg samples were weighed individually, and were stored at 4˚C. Afterward, the egg quality parameters were calculated using routine methods.

2.3. Collection of Samples

At the end of the experiment (49 d), 6 hens per treatment (2 hens per replicate) were randomly selected and euthanized by cervical dislocation. Then, the whole intestinal tract was immediately excised and put in sterile bags, placed on ice to take to the laboratory for further sampling.

2.4. Microbial Analysis

The two ceca were longitudinally opened using a pair of sterile scissors, and digesta samples (1 g) were collected after both ceca were cut down. The samples were serially diluted from 10-1 to 10-7 in sterilized physiological saline solution. Selective agar media was used for enumeration of *Escherichia coli* (MacConkey Agar, Hopebio, Qingdao, China), *Salmonella* (*Salmonella* Shigella Agar, Hopebio, Qingdao, China), *Lactobacillus* (*Lactobacillus* Selection, Hopebio, Qingdao, China), and *Bifidobacterium* (Bismuth Sulfite Agar, Hopebio, Qingdao, China) by conventional microbiological techniques. Briefly, triplicate plates were inoculated with 1 mL samples, and three suitable dilutions were plated for each medium. *Escherichia coli* and *Salmonella* were enumerated on agar after aerobic incubation at 37˚C for 18 - 24 h. *Lactobacillus* and *Bifidobacterium* were enumerated after anaerobic chamber incubation at 37˚C for 48 h. In addition, the numbers of colony-forming units in the duplicate plates were averaged. The cecal contents were denoted as base 10 log colony-forming units per gram (cfu/g).

2.5. Digestive Enzymes Activity Assay

The intestinal digesta samples were collected by scraping the tract gently and weighed. Then, the digesta samples were immediately placed into liquid nitrogen and stored at −80˚C until further analysis. Thawed digesta samples were diluted 10× using ice cold PBS (pH 7.0) based on the sample weight. After being fully homogenized, the samples were centrifuged at 2500 rpm for 8 - 10 min, and the supernatants were stored at −20˚C for enzyme assays.

Amylase, lipase and trypsin activity were detected with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) via spectrophotometry (UV-2102C, UNICO, Shanghai, China), a simplified turbidimetric assay and ethyl N-benzoyl-L-argininate hydrochloride (BAEE) as a substrate were performed according to the manufacturer's instructions, respectively. Enzyme activity was described using enzymatic activity units (U) per gram.

2.6. Intestinal Morphology

Segments (1 cm) of the duodenum (from the gizzard to pancreatic), jejunum (between the entry point of bile ducts and Meckel's diverticulum) and ileum (5 cm proximal to the ileocecal junction) were all removed from the whole intestinal tract. The samples were fixed in 4% formalin after washing with cold physiological saline. Each sample was embedded in paraffin wax, and tissue sections (5 μm) were cut before staining with hematoxylin and eosin, and villus height (from the tip to the villus crypt junction) and crypt depth (from the valley between adjacent villi to the basolateral membrane) were examined by optical microscopy with a digital video camera (B5 Digital, Motic, Xiamen, China). Morphological indices were measured using stereological image software (Med6.0, Motic, Xiamen, China). And morphological indices data were analyzed with SPSS 17.0 software.

2.7. Real-Time PCR

Segments of the duodenum and jejunum were opened longitudinally and rinsed with

ice cold normal saline, scraped with glass slides, the intestinal mucosa frozen in liquid nitrogen, and stored at −80˚C. Total RNA was extracted from intestinal tissues using a QIAGEN RNeasy Mini Kit (Hilden, Germany), according to the manufacturer's instructions. The purity and concentration of the total RNA were measured at 260/280 nm with a SmartSpecTMPlus Spectrophotometer (BIO-RAD, California, USA). The cDNA was synthesized from 1 μg of total RNA using a PrimeScript® RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's protocols. The products (cDNA) were stored at −20˚C for RT-PCR. The specific primers for sodium-glucose cotransporter 1 (SGLT1), glucose transporter 2 (GLUT2), peptide transporter 1 (PepT1) and *β*-actin(ACTB) were designed using Primer 5.0 (Table 3). PCR amplification was conducted, and the products were checked on 1% agarose gels, extracted, cloned into the pMD18-T vector (Takara) and sequenced. A standard curve was made of serially diluted plasmid by plotting threshold cycle values. RT-PCR was performed using SYBR® Premix Ex Taq™ (Takara) and 1 μL cDNA on a Line-gene K Real-Time PCR Detection System with software (Bioer Technology, Hangzhou, China) in 50 μL reactions. Thermal cycling was performed with the following conditions: 94˚C for 3 min and 45 cycles of 94˚C for 15 s, 57˚C for 30 s, and a final step of 72˚C for 20 s. Each sample was measured for three replicates and normalized by using *β*-actin. The samples' copy number was calculated by interpolating the threshold cycle values with the standard curve.

2.8. Statistical Analysis

All data were analyzed with SPSS 17.0 software (SPSS Inc, Chicago, IL, USA). The statistically significant differences in treatment means were evaluated using one-way ANOVA, and a multiple comparison (Duncan)test was conducted with significant treatment means. $P < 0.05$ was considered as the significant level and $P < 0.01$ as extremely significant. Values are expressed as the means ± standard deviation.

3. Results

3.1. Production Performance

The effects of oregano essential oils supplementation in the diet of laying hens on the production performance of five different groups (NC, CS, I, II and III) are presented in

Table 3. List of primers used for real-time PCR.

Target	Accession No.	Primer sequence (5'-3')	Amplicon length (bp)
SGLT1	XM_415247	F: CCTGGAATGATCAGCCGAAT R: ACCTCGTAGACCATTTGGCATAA	150 bp
GLUT2	NM_207178	F: GGCGTTGGAGTGGTGAACAC R: GAACTGGCTCAGGAGCACAAG	150 bp
PepT1	AY029615	F: CCCCAATTCTCAGAGCTCAAGA R: TGCCTTGCGGTTGAACTTTT	150 bp
ACTB	NM205518	F: GTTGACAATGGCTCCGGTAT R: TGGGCTTCATCACCAACGTA	150 bp

Table 4. The addition of 100 mg/kg oregano essential oils significantly increased the laying rate ($P < 0.01$), average egg weight, and improved feed conversion ratio ($P < 0.01$) compared with the NC and CS groups. However, the daily intake was statistically similar among dietary treatments ($P > 0.05$). Morbidity and mortality were nil in all treatments.

3.2. Egg Quality

Egg weight, eggshell ratio, yolk index and Haugh units were not influenced significantly by oregano treatments compared with the NC and CS groups ($P > 0.05$) (Table 5). However, 50 mg/kg oregano essential oils diet increased the percentage of yolk ratio and egg shape index ($P < 0.05$) compared with the CS group, but there was no significant difference in oregano treatments compared with the NC group ($P > 0.05$).

3.3. Cecal Microflora

The results of cecal flora analysis are summarized in Table 6. In the cecal digesta, *Bifidobacterium* and *Lactobacillus* counts were significantly ($P < 0.01$) increased for hens fed 100 mg/kg of oregano essential oils versus other groups, whereas *Escherichia coli* and *Salmonella* counts were significantly ($P < 0.01$) decreased, ever lower than in the CS group. The present study demonstrated that supplementation of oregano essential oils has a positive effect on intestinal microbiology.

Table 4. Effect of oregano essential oils supplementation on performance of laying hens.

Parameter	Groups				
	NC	CS	I	II	III
Laying rate (%)	91.97 ± 3.35^{Bb}	92.43 ± 2.51^{Bbc}	94.50 ± 2.69^{ac}	96.88 ± 1.55^{Aa}	95.09 ± 1.48^{a}
Average egg weight (g)	61.11 ± 1.34^{b}	60.14 ± 1.56^{Bab}	62.65 ± 1.07^{Aab}	63.23 ± 2.12^{Aa}	61.66 ± 1.81^{ab}
Daily feed intake (g)	141.63 ± 3.98	141.98 ± 3.89	138.89 ± 4.58	141.29 ± 3.40	142.55 ± 2.19
Feed conversion ratio (g feed/g egg)	2.53 ± 0.17^{B}	2.54 ± 0.12^{B}	2.35 ± 0.12^{A}	2.31 ± 0.12^{A}	2.43 ± 0.10^{AB}

Values on the same line sharing a different superscript a, b, c are significantly different at $P < 0.05$. Values on the same line sharing a different superscript A, B, C, D are significantly different at $P < 0.01$. *NC (no treatment), CS (10% colistin sulfate, 100 mg/kg diet), I (oregano essential oils, 50 mg/kg diet), II (oregano essential oils, 100 mg/kg diet), and III (oregano essential oils, 150 mg/kg diet). Same as below.

Table 5. Effect of oregano essential oils supplementation on egg quality parameters of laying hens.

Parameter	Groups				
	NC	CS	I	II	III
Egg weight (g)	64.18 ± 4.31^{ab}	62.62 ± 5.98^{ab}	60.90 ± 4.54^{a}	65.75 ± 5.60^{b}	64.34 ± 4.23^{ab}
Eggshell ratio (%)	9.86 ± 1.45	9.75 ± 0.78	10.22 ± 1.08	10.67 ± 1.79	10.43 ± 1.43
Yolk ratio (%)	32.84 ± 1.38^{ab}	31.37 ± 0.86^{bc}	34.16 ± 3.33^{Aa}	33.00 ± 2.93^{ab}	30.42 ± 1.53^{Bc}
Egg shape index (%)	128.78 ± 0.06^{ab}	126.89 ± 0.05^{b}	132.67 ± 0.06^{a}	130.56 ± 0.03^{ab}	130.56 ± 0.04^{ab}
Yolk index (%)	41.40 ± 1.15	40.92 ± 10.88	41.28 ± 8.00	41.76 ± 3.56	43.68 ± 4.95
Haugh units (%)	78.91 ± 2.15^{ab}	80.37 ± 3.42^{ab}	76.84 ± 1.31^{a}	78.80 ± 2.39^{ab}	81.79 ± 3.58^{b}

Table 6. Effect of oregano essential oils supplementation on cecum flora of laying hens (cfu/g).

Microflora	Groups				
	NC	CS	I	II	III
Lactobacilli	6.09 ± 0.14^{CD}	6.20 ± 0.16^{ADb}	6.42 ± 0.11^{Aa}	7.09 ± 0.11^{B}	6.88 ± 0.05^{B}
Bifidobacteria	7.26 ± 0.09^{C}	7.53 ± 0.12^{A}	7.56 ± 0.07^{A}	8.02 ± 0.15^{B}	7.67 ± 0.08^{A}
Escherichia coli	7.02 ± 0.07^{C}	6.39 ± 0.07^{Aa}	6.40 ± 0.07^{Aa}	6.21 ± 0.07^{Bb}	6.25 ± 0.06^{ABb}
Salmonella	5.80 ± 0.12^{C}	5.3 ± 0.10^{B}	5.04 ± 0.06^{Aa}	4.80 ± 0.08^{Ab}	4.90 ± 0.10^{A}

3.4. Intestinal Morphology

The morphology data indicated that the addition of 100 mg/kg oregano essential oils increased the villus height ($P > 0.05$) and villus-height-to-crypt-depth ratios ($P < 0.01$) in the duodenum compared with the NC group (Table 7). The duodenum crypt depth was decreased by 100 mg/kg oregano essential oils supplementation compared with the NC group. However, for the jejunum, after the addition of oregano essential oils, there was a slight effect on villus height, crypt depth or villus-height-to-crypt-depth ratios in comparison with the NC and CS groups ($P > 0.05$).

3.5. Digestive Enzyme Activities

The effects of oregano essential oils supplementation on intestinal digestive enzyme activities in laying hens are shown in Table 8. The addition of 100 mg/kg oregano essential oils on hens produced a significant increase in the activities of amylase and trypsin relative to the NC group ($P < 0.01$), and amylase activity was higher ($P < 0.05$) in the group II than in the CS group. Lipase activity was slightly increased by dietary treatments ($P > 0.05$). The results indicated that supplementation with oregano essential oils improved the activities of digestive enzyme activities and may improve the digestion of nutrients in the small intestine.

3.6. Glucose and Peptide Transport Gene Expression

The effect of oregano essential oils supplementation on GLUT2, PepT1 and SGLT1 mRNA expression data are presented in Table 9. A significant increase ($P < 0.01$) in the GLUT2, PepT1 and SGLT1 gene expression could be observed in group II, which received the diet containing 100 mg/kg oregano essential oils, in the duodenum and jejunum. The increase in these parameters corresponds to an increase in nutrition absorption.

4. Discussion

Oregano essential oils has been developed as an antibiotic alternative in the poultry industry partly because of biological activities, such as antimicrobial, antioxidant, antiseptic and antiparasitic activity. Some researchers have obtained positive results concerning the efficacy of oregano essential oils on the performance in poultry, which is in agreement with our results. Radwan *et al.* have reported that the use of 1.0% oregano in the diet increased egg production and egg weight and improved feed conversion of hens [11]. Roofchaee *et al.* obtained a similar result in that dietary oregano essential oils

Table 7. Effect of oregano essential oils supplementation on the intestinal morphological parameters of laying hens.

Parameters	Groups				
	NC	CS	I	II	III
Duodenum					
Villus height (μm)	385.99 ± 5.88	389.92 ± 4.27	386.94 ± 2.05	394.10 ± 4.36	393.30 ± 5.16
Crypt depth (μm)	34.48 ± 0.47^{b}	33.40 ± 0.98^{ab}	33.35 ± 0.58^{ab}	33.18 ± 0.37^{a}	33.53 ± 0.60^{ab}
Villus height/crypt depth	11.20 ± 0.28^{Bb}	11.68 ± 0.39^{a}	11.60 ± 0.15^{ab}	11.88 ± 0.04^{Aab}	11.73 ± 0.30^{a}
Jejunum					
Villus height (μm)	319.05 ± 3.17	322.02 ± 7.30	317.43 ± 5.97	330.99 ± 17.77	327.86 ± 8.80
Crypt depth (μm)	29.52 ± 0.83	29.28 ± 0.68	29.04 ± 0.37	28.27 ± 0.94	28.60 ± 1.01
Villus height/crypt depth	10.81 ± 0.33	11.00 ± 0.33	10.93 ± 0.33	11.73 ± 0.96	11.47 ± 0.32

Table 8. Effect of oregano essential oils supplementation on the digestive enzyme activities in the small intestinal contents of laying hens (U/mg protein).

Enzyme	Groups				
	NC	CS	I	II	III
Amylase	4.65 ± 0.10^{A}	4.73 ± 0.16^{b}	4.64 ± 0.08^{A}	4.99 ± 0.13^{Ba}	4.76 ± 0.14^{b}
Lipase	75.60 ± 0.46	75.62 ± 0.46	75.91 ± 0.35	76.11 ± 0.15	76.03 ± 0.17
Trypsin	1964.26 ± 69.77^{A}	2074.87 ± 94.68^{AB}	1967.44 ± 47.54^{A}	2160.10 ± 80.22^{B}	2042.31 ± 71.41^{AB}

Table 9. Effect of oregano essential oils supplementation on the GLUT2, SGLT1, and PepT1 gene expression in the intestines of laying hens.

Parameters	Groups				
	NC	CS	I	II	III
1-Duodenum					
GLUT2 (AU)	1.00 ± 0.59^{Aa}	1.56 ± 0.36^{Aac}	2.05 ± 0.92^{Aac}	6.02 ± 0.67^{B}	2.33 ± 0.18^{Abc}
SGLT1 (AU)	1.00 ± 0.16^{Aa}	3.08 ± 0.95^{CDb}	1.90 ± 0.45^{ACa}	6.19 ± 0.53^{B}	3.36 ± 0.32^{Db}
PepT1 (AU)	1.00 ± 0.26^{Aa}	3.56 ± 0.92^{Ca}	1.55 ± 0.21^{Aa}	7.18 ± 0.97^{B}	3.91 ± 0.23^{Ca}
2-Jejunum					
GLUT2 (AU)	1.00 ± 0.40^{Aa}	1.01 ± 0.33^{Aa}	0.88 ± 0.35^{Aa}	3.39 ± 0.22^{B}	0.28 ± 0.04^{Ab}
SGLT1(AU)	1.00 ± 0.30^{Aa}	1.28 ± 0.52^{a}	0.47 ± 0.02^{Aa}	2.35 ± 0.86^{Bb}	1.28 ± 0.22^{a}
PepT1 (AU)	1.00 ± 0.25^{Aa}	3.53 ± 0.67^{Ca}	2.02 ± 0.29^{Ab}	5.23 ± 0.66^{B}	2.51 ± 0.68^{Cb}

significantly increased body weight and improved the feed conversion ratio of broiler chickens [8]. The beneficial effect of oregano essential oils may be attributed to the antimicrobial activity of phenolic compounds [13]. Lee *et al.* indicated that carvacrol, a component of essential oils, improved the FCR of broilers, and this could be connected with the increase of feed utilization efficiency [14]. Furthermore, Windisch *et al.* reported that phytogenic compounds may specifically enhance the activities of digestive enzymes and nutrient absorption [3]. Therefore, dietary feeding of oregano essential

oils may improve the intestinal healthy and digestibility of the feeds and thus improve the performance of laying hens.

Furthermore, it is important to consider that several phytogenic product feed additives have a potential impact on the intestinal microflora either directly or indirectly [15]. In the current study, oregano essential oils supplementation of diets demonstrated a beneficial effect on the gut flora of laying hens. These findings are in compliance with the previous reports [7] [16]. These researchers found that oregano had antibacterial activity against E. coli and *Salmonella*. The broad antimicrobial activity may be due to the presence of thymol and carvacrol, the major active components of oregano essential oils. Previous studies have indicated that the antibacterial mechanism of carvacrol and thymol may be similar in that they increase the permeability of the cytoplasmic membrane by disrupting bacterial outer membranes, which leads to ATP leaking out of the cell. Carvacrol can also inhibit ATPase and thus be able to influence the proliferation of bacteria [17]. Moreover, carvacrol also has a stimulating effect on *Lactobacillus* proliferation. In the present study, the dietary treatments also significantly increased the *Lactobacillus* counts.

In addition, it is reasonable to study digestive enzymes existing in the small intestine and pancreas to reveal the effects of oregano essential oils on digestive function, as major nutrient digestive processes occur in these areas. It has been reported that the addition of essentialoilss including thymol, one of the main components of oregano essential oils, markedly increased the amylase activity in the intestinal digesta at d 21 in broilers [14]. Similar to our results, Jamroz *et al*. observed the use of plant extracts containing carvacrol and other active substances enhanced the activities of pancreas α-amylase and α-amylase and lipase in the intestine tissues of older chickens [18]. Similarly, Hashemipour *et al*. proved that carvacrol and thymol could increase intestinal trypsin and lipase activities in broilers at 24 d of age [19].

Data from the intestinal flora have demonstrated that oregano essential oils exerted a positive impact on *Lactobacillus* and *Bifidobacterium* in the cecum. Such changes in intestinal microbiology with oregano essential oils might contribute to the observed effects on digestive enzyme activities. Previous studies have reported that the *Bifidobacterium* and *Lactobacillus* colonizing the intestine can deliver enzymes, which then leads to increased digestive enzyme activity in the intestines [20]. Moreover, Hashemipour *et al*. also postulated that the use of phytogenic product would stimulate the secretion of intestinal digestive enzymes and enhance the digestion of nutrients under certain circumstances [19]. Therefore, the results reported here indicate that the addition of oregano essential oils to the diet of laying hens enhanced intestinal digestive function.

Both crypt depth and villous height are important indicators of chicken digestive health and related to the absorptive capacity of intestinal mucous membrane directly. Moreover, the villous: crypt ratio is likely an indicator of the digestive capacity of gut. Our results are in compliance with the research of Perić *et al*. who found that adding phytogenic additives in the diet of 42-day-old chickens resulted in an increase in the villous height [21]. Similarly, Betancourt *et al*. demonstrated that diet supplementation with 200 ppm active essential *O. vulgare* L. resulted in a significant increase in villous height at 3 days and slightly decreased crypt depth and increased the villous: crypt ratio in 7-day-old

chickens [22]. Our study revealed a significant increase of the villous: crypt ratio. These findings indicate that the addition of oregano essential oils to the diet of laying hens enhanced the digestive and absorptive capacity of the intestinal mucous membrane.

The absorption of glucose is mediated by SGLT1, in a Na^+-dependent manner, which transports glucose and galactose into the enterocyte [23]. In addition, the exit of glucose, galactose and fructose across the basolateral membrane is facilitated by the transporter GLUT2, which is dependent on the transmembrane concentration gradient [24]. PepT1 is the peptide transporter, which transports divides- and tripeptides into the cell. The PepT1 pathway is a major mechanism for the absorption of intestinal products of protein digestion. Gilbert demonstrated that gene expression of the nutrient transporter is responsive to various factors, including dietary manipulation, genetic selection, and developmental stage [25]. These strategies can be used to enhance nutrient utilization by improving dietary formulation, leading to improved chicken performance. Our study found that the addition of 100 mg/kg oregano essential oils resulted in a significant increase in SGLT1, GLUT2 and PepT1 mRNA expression, which improved the glucose and protein absorption capacity. A previous study reported that long-term luminal glucose exposure can increase SGLT1 activity in enterocytes and dipeptides themselves, presumably by stimulating gene expression of PepT1 to promote increased dipeptide transport [26]. Therefore, the increase in SGLT1, GLUT2 and PepT1 mRNA expression in the present study may be due to the increased concentration of glucose and peptide in the gut. Furthermore, the nutrition increase may be related to the improved digestive enzyme activity and intestinal morphology.

Colistin (polymyxin E) is a polymyxin antibiotic produced by certain strains of Paenibacillus polymyxa var. colistinus. Recently, the researchers found evidence of transferable resistance to the polymyxin drug colistin in bacteria isolated from pig and poultry in China [27]. The Chinese government has launched a risk assessment on the use of colistin in animal feed and will limit or stop using polymixins in agriculture. Thus, we chose colistin as an antibiotic control. The results proved that dietary inclusion of oregano essential oils increased the laying performance and improved gut microflora, intestinal morphology and nutrient absorption in laying hens. Therefore, oregano essential ilia an ideal herbal antibiotic alternative in the laying hens.

5. Conclusion

In conclusion, oregano essential oil enhanced average egg weight and feed efficiency on laying hens. And the additive increased the number of intestinal *Bifidobacterium* and *Lactobacillus and* the number of intestinal *Escherichia coli* and *Salmonella* decreased. Also the additive increased digestive enzyme activities and improved the intestinal morphology. Furthermore, the gene expression levels of GLUT2, PepT1 and SGLT1 in the duodenum and jejunum were significantly increased. Dietary inclusion of oregano essential oils increased the laying performance and improved nutrient absorption in laying hens.

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