Dietary Oregano Essential Oil Supplementation Combined with Cinnamaldehyde Improves Production Performance and Alters Gut Microbiota in Late-Phase Laying Hens

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**Dietary oregano essential oil supplementation combined with
cinnamaldehyde improves production performance and alters gut
microbiota in late-phase laying hens**

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Abstract

Background: Essential oils (EO) have the characteristics of antibacterial and anti-inflammatory, and are called natural antibiotics. This study was aimed to investigate the potential effects of EO on production performance, egg quality, fatty acid composition in yolk and cecum microbiota of hens in the late phase of production. A total of 350 58-week-old Jing Tint 6 laying hens were randomly divided into 5 groups: 1) a basal diet (control); 2) a basal diet + 5 mg/kg flavomycin (AGP); 3) a basal diet + 100 mg/kg oregano essential oil (OEO) + 20 mg/kg cinnamaldehyde (EO1); 4) a basal diet + 200 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde (EO2); 5) a basal diet + 300 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde (EO3).

Results: Compared to the control group, EO2 group resulted in a higher ($P < 0.05$) egg production during weeks 5-8 and 1-8. EO2 group had a lower feed conversion ratio than control group during weeks 1-8. The content of monounsaturated fatty acid (MUFA) in EO2 group is higher ($P < 0.05$) than that of control and AGP groups. EO2 group increased ($P < 0.05$) the abundance of Actinobacteriota and decreased the abundance of *Desulfovibrio* in cecum. The abundances of *Anaerofilum*, *Fournierella*, *Fusobacterium* and *Sutterella* were positively correlated with egg production, feed conversion ratio, and average daily feed intake, while the abundances of *Bacteroides*, *Desulfovibrio*, *Lactobacillus*, *Methanobrevibacter*, and *Rikenellaceae_RC9_gut_group* were negative correlated with egg production, feed conversion ratio, and average daily feed intake.

Conclusions: Dietary OEO at 200 mg/kg and cinnamaldehyde at 20 mg/kg addition could improve the egg production performance, decrease feed conversion ratio and alter the egg fatty acid and microbial composition of late-phase laying hens.

37 **Keywords:** Essential oils; Production performance; Fatty acid composition; Bacterial
38 community; Laying hen
39

Introduction

Antibiotics have been widely used in poultry industry as animal growth promoters for decades. However, the long-term sub-therapeutic antibiotics caused a series of problems, such as making bacteria resistant to antibiotics, antibiotic residues, endogenous infections and double infections which pose serious threats to livestock and poultry production and human health[1,2]. Now, China has banned feed antibiotic, leading to increased demand for green feed additives.

Essential oils (EO) are concentrated hydrophobic liquids containing volatile aromatic compounds obtained from plants[3]. EO have strong antibacterial properties on various pathogenic and bacteria, such as *Salmonella enteritidis*, *Escherichia coli*, *Staphylococcus aureus*, *Campylobacter*, etc. [4,5], which are considered as a substitute for antibiotics. EO or their main components (thymol and carvacrol) can destroy the cell membrane structure to exert antibacterial activity[3,6]. Furthermore, EO has been widely used in poultry and pigs for many years. The supplement of EO (containing thymol and carvacrol) could alleviate the intestinal injury in broiler chickens under *Clostridium perfringens* challenge [7]. EO (carvacrol as main component) may enhance the growth performance and intestinal health of broilers[8-10]. EO (containing thymol and carvacrol or cinnamaldehyde) not only inhibit the growth of pathogen bacteria, but modulate intestinal microbial composition of broilers[11,12]. The efficacy of EO (thymol, carvacrol or menthol as active components) on improving laying performance and egg quality has been reported[13-15]. Cinnamaldehyde is the major compounds of cinnamon essential oil, which can alter the microbial composition[16]. It is reported that cinnamaldehyde could enhance growth performance by improving intestinal histomorphology in broilers under a necrotic enteritis challenge[17]. Dietary supplementation of cinnamaldehyde could improve nutrient utilization and decrease total nitrogen excretion in

broiler[18]. However, the effects of OEO and cinnamaldehyde on the production performance, egg quality and intestinal microbial community of laying hens in the late phase of production await further studies.

In this study, we hypothesized that the OEO and cinnamaldehyde may serve as antibiotic alternatives to positively alter the microbial composition, subsequently conducting to the improvements of production performance, egg quality and yolk fatty acid composition. Therefore, the purpose of this study was to investigate the effects of dietary oregano essential oil and cinnamaldehyde supplementation on laying performance, egg quality, fatty acid composition in yolk, and microbial community in the cecum of laying hens in the late phase of production.

Materials and methods

Animals and experimental design

All experimental protocols involving animals in this study satisfied animal welfare and approved by Shandong University of Traditional Chinese Medicine. A total of 350 58-week-old Jing Tint 6 laying hens were randomly divided into 5 groups with 5 replicates of 14 birds: 1) a basal diet (control); 2) a basal diet + 5 mg/kg flavomycin (AGP); 3) a basal diet + 100 mg/kg oregano essential oil (OEO) + 20 mg/kg cinnamaldehyde (EO1); 4) a basal diet + 200 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde (EO2); 5) a basal diet + 300 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde (EO3). The birds were provided by Gu'an Songhe Poultry Breeding Co. Ltd (Hebei China). OEO product (containing 8% oregano essential oil) was obtained from a commercial supply (Ropapharm International B.V. Zaandam Netherlands) and its active ingredients were carvacrol and thymol, with wheat flour and silicon dioxide as carrier. The actual concentrations of oregano EO in the EO1, EO2 and EO3 diets were 8, 16 and 24 mg/kg, respectively. The concentration of carvacrol

and thymol in EO was $\geq 3.6\%$ and $\geq 0.1\%$, respectively. The cinnamaldehyde was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). All birds were housed in four-tier battery cages in an environmentally controlled house with temperature maintained at approximately 23 °C and humidity maintained in 30%-48%. All birds were fed with the same wheat-soybean meal basal diet and provide 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) recommendation. All birds remained in good health and medical intervention was not applied to any birds during the whole feeding period.

Sample collection

Three eggs per replicate were randomly collected for egg quality determination every 4 weeks. Two eggs in each replicate were randomly sampled on day 56 to separate egg yolk from egg whites. Egg yolk was freeze-dried for fatty acid determination. At the end of study, 1 bird per replicate (5 per treatment) was randomly selected to slaughter after fasting for 12 h. Cecal contents were collected and kept frozen at $-80\text{ }^{\circ}\text{C}$ for microbial composition.

Laying performance and egg quality

During the experimental period, egg production, broken egg production and egg weight were recorded daily by replicate and feed consumption for each replicate was weighted every week. Feed conversion ratio (FCR) was calculated as grams of feed consumption/egg weight for each replicate. 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 (EFR-01, Israel Orka Food Technology Ltd.) and Egg Shell Thickness Gauge (ESTG-1, Israel Orka Food Technology Ltd.). Egg weight, haugh unit values and albumen height were measured by an

Egg Analyzer (EA-01, Israel Orka Food Technology Ltd.).

Analysis of ileal microbiota

Total genome DNA from samples was extracted using CTAB method. DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1ng/μl using sterile water. The V3-V4 region of the 16S rRNA gene was amplified using the primer pair 341F/806R (5'-CCTAYGGGRBGCASCAG-3' and 5'-GGACTACHVGGGTWTCTAAT-3'). Sequencing libraries were generated using Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated. Sequencing and bioinformatics analysis were performed by Novogene Bioinformatics Technology Co. (Beijing, China).

Statistical analysis

Data were analyzed by one-way Analysis of Variance (ANOVA) procedure and differences were examined using Duncan's Multiple Range Test using IBM SPSS Statistics 23. The differences in the relative abundances of bacteria between groups were assessed using Wilcoxon rank tests. Data were presented as mean ± standard deviation and statistical significance was defined as a *P* value < 0.05.

Results

Laying performance and egg quality

Dietary EO supplementation had no significant influences (*P* > 0.05) on average daily feed intake (ADFI), and broken egg production of laying hens during weeks 1-4, 5-8, and 1-8 of the experiment (Table 2). However, EO2 increased (*P*<0.05) egg production of laying hens in comparison with

control in weeks 5-8 and 1-8. Average egg weight in EO2 group was lower ($P < 0.05$) than that in the AGP in weeks 1-4, but it was higher ($P < 0.05$) than that in the control during weeks 5-8 and 1-8. The EO2 group decreased the FCR when compared to the control and AGP groups, although the difference is not clear significant ($P > 0.05$). In egg quality, there was no significant effects ($P > 0.05$) of dietary EO and cinnamaldehyde supplementation on eggshell strength, shape index, albumen height, and Haugh unit at the end of week 4 and 8 (Table 3). Eggshell thickness in EO2 group was lower ($P < 0.05$) than that in the control and AGP in week 4.

Yolk fatty acids composition

Fatty acids composition and content of yolks at week 8 are showed in Table 5. SFA, UFA, PUFA, EFA, n-3 PUFA, and n-6 PUFA had no difference among treatments at week 8. However, the concentration of MUFA in EO2 and EO3 groups was higher ($P < 0.05$) than that in the control and AGP groups at week 8.

Cecal microbial profile

As shown in Table 6, the Chao1 index of the cecal microbiota in layers with EO2 was higher ($P < 0.05$) than that in the AGP group. There was no significant difference of dietary EO supplement on Shannon, Simpson, and ACE than that in control and AGP group. The ASVs Venn analysis identified 387, 315 and 764 unique ASVs in control, AGP and EO2 group, respectively. Three treatments shared 53 ASVs among their cecum microbiota (Fig. 1A). The PLS-DA results suggested that there was a clear difference in the bacterial community among three treatments (Fig.1B).

The dominant phyla in the three treatment groups were Bacteroidota and Firmicutes, together occupying more than 75% of whole phyla (Fig.2A). Laying hens supplemented with EO2 had a higher abundance of Bacteroidota and a lower abundance of Firmicutes compared to AGP group.

At the class level, the dominant classes were Bacteroidia, Clostridia and Fusobacteriia, which collectively occupied more than 73% of the total sequences (Fig.2B). At the family level (Fig.2C), the cecal microbiota was dominated by Bacteroidaceae in all groups.

At the phylum level, the abundance of Desulfobacterota in control group was higher ($P < 0.05$) than that of AGP and EO2 groups, and EO2 had higher ($P < 0.05$) abundance of Actinobacteriota than that of the control and AGP groups (Table 7). At the class level, the control had higher ($P < 0.05$) abundances of Desulfovibrionia, than that in AGP and EO2. At the family level, AGP had higher ($P < 0.05$) abundances of Lachnospiraceae than the control and EO2, and the control had higher ($P < 0.05$) abundances of Desulfovibrionaceae than AGP and EO2 groups. At the genus level, control had higher ($P < 0.05$) abundances of *Desulfovibrio* than AGP and EO2 groups.

Correlation between cecal microbiota and laying performance

As shown in Fig. 4, the egg production was positively correlated with the abundances of *Sutterella* and *Anaerofilum*, but it was negative correlated with *Lactobacillus*, *Methanobrevibacter*, *Desulfovibrio* and *Rikenellaceae_RC9_gut_group* abundances. FCR was positive correlated with *Fusobacterium*, *Fournierella*, *Sutterella* and *Anaerofilum*, while showed a negative correlation with *Bacteroides*. There was a positive correlation between ADFI and *Anaerofilum*, *Sutterella* and *Fournierella*, and a negative correlation between ADFI and *Lactobacillus*, *Methanobrevibacter*, *Rikenellaceae_RC9_gut_group*, *Desulfovibrio* and *Bacteroides*.

Discussion

Essential oils and their active ingredients have been extensively studied for their growth-promoting properties, which make them potential alternative to AGPs[19]. In the present study, egg production was improved in the EO2 treatments, but there was no significant effect on FCR in EO groups

172 compared to control and AGP groups. In line with our study, several previous studies had reported
173 that EO improved egg production in laying hens[20,21]. The beneficial effect could be attributed to
174 the active ingredients (thymol and carvacrol) in OEO and cinnamaldehyde, which have been proven
175 to have antibacterial, anti-inflammatory activity and improve intestinal health and nutrient
176 utilization[18,22]. In contrast, other studies had shown that EO or their main compounds did not
177 significantly improve laying performance of laying hens[23,24]. The inconsistent effects of EO on
178 production performance may be caused by the composition and supplemental levels of EO, the type
179 of laying hen, the feeding phase, and the environmental conditions. It is noteworthy that FCR of
180 EO2 treatment was lower ($P < 0.05$) than that of EO1 and EO2 groups, which may be due to the
181 negative effects of high concentrations of essential oils on intestinal epithelial cells and intestinal
182 probiotics[25,26].

183 Eggshell thickness is an important indicator of egg quality and is critical for the egg transportation
184 and storage[27]. During eggshell calcification, the availability of intestinal calcium is very vital as
185 it plays a key role in providing sufficient amounts of calcium to satisfy shell quality requirement[28].
186 It has been reported that dietary essential oils could significantly increase the eggshell
187 thickness[20,29]. In our study, the eggshell thickness of EO1 and EO2 was lower ($P < 0.05$) than
188 that of control and AGP at week 4, but the difference disappeared at week 8, which may be due to
189 altered flora structure[30].

190 The lipid composition of eggs has received attention due to the relationship between dietary
191 lipids and the development of coronary heart disease[31]. MUFA have a significant impact on
192 improving cardiovascular and cerebrovascular diseases, diabetes, and reducing oxidative stress
193 damage in the body[32]. Some countries even advocated that MUFA should become healthy

substitutes in the daily diet. In addition, MUFA have physiological functions on the body such as lowering blood sugar, regulating blood lipids, and lowering cholesterol[33,34]. The previous research reported that dietary EO or rosemary extract supplementation in layer diet did not significantly influence fatty acid composition of egg yolk of laying hens[20,35]. Another study found that dietary bergamot oils could significantly increase the proportion of DHA and the n-3 proportion of egg yolk [36]. In the present study, the content of MUFA of EO2 and EO3 was higher ($P < 0.05$) than that of the control and AGP treatments, which may be due to OEO and cinnamaldehyde affecting lipid metabolism in serum and liver of laying hen[36,37].

Intestinal flora is not only involved in food digestion and metabolism, body growth and development, immune suppression and activation, but also one of the three major components of the intestinal barrier, which is a direct factor in whether pathogenic bacteria can invade[38-40], so the further study was performed to better understand the effect of OEO and cinnamaldehyde on the microbiota. Since the beneficial effects of OEO combined with cinnamaldehyde on production performance were mainly observed in the EO2 group, the modulatory roles of EO on cecal microbial composition were assessed in the control, AGP and EO2 groups. ACE and Chao1 indices were used to estimate species richness, while Shannon and Simpson indices were used to estimate species diversity[41]. The Chao1 of EO2 was higher ($P < 0.05$) than that of AGP group, indicating that the combination of OEO and cinnamaldehyde would increase the abundance of cecal flora. According to the beta-diversity results of three treatments, the significant clustering was observed, indicating that the addition of OEO and cinnamaldehyde altered the cecal microbial community structure. Then, further analysis of changes in microbiota composition and specific taxa upon addition of OEO and cinnamaldehyde was performed. At the phylum level, Bacteroidota and Firmicutes, as dominant

flora, accounted for more than 75% of the total microbial community. Firmicutes and Bacteroidetes are involved in the metabolism of nutrients in the body, such as enzymes encoding polysaccharide decomposition, involved in sugar metabolism, and cooperatively involved in amino acid metabolism[42,43]. Actinobacteriota is reported to improve feed utilization by producing extracellular enzymes[44], and decompose undigested components in feed through secreting endogenous enzymes[45]. In addition, Actinobacteriota is helpful for the maintenance of overall microbial structure. Due to the production of bacteriocins and the ability to convert feed into fermentable microbial biomass[46], Actinobacteriota is considered to be a key group that regulates the function of the gut microbiota. In the present study, the abundance of Actinobacteriota in EO2 group was higher ($P < 0.05$) than that of the control and AGP, indicating that dietary supplementation OEO and cinnamaldehyde may increase the abundance of beneficial bacteria to promote the production performance and feed utilization of the late laying hens. Short chain fatty acids (SCFAs) play important roles in the gut, such as anti-inflammation, cancer prevention, clearance of drug-resistant pathogenic bacteria and regulation of gene expression[47-49]. Studies had shown that Lachnospiraceae can promote SCFAs production[50]. At the family level, the abundance of Lachnospiraceae in AGP was higher ($P < 0.05$) than that of the control and EO2, speculating that AGP might promote production performance by increasing SCFA producing bacteria, but the specific mechanism of action needs to be studied in depth. At the genus level, the abundance of *Desulfovibrio* in the AGP and EO2 was lower ($P < 0.05$) than that of the control. *Desulfovibrio* are considered as harmful bacteria, because *Desulfovibrio* acts as a hydrogen sink in the cecal ecosystem of birds, and hydrogen inhibits the production of short-chain fatty acids[51,52]. Therefore, *Desulfovibrio* was highly negatively correlated with laying hens' egg production and

ADFI in our study. *Lactobacilli* is considered as beneficial bacteria due to reduce colonization by enteric pathogens through competitive exclusion, antagonistic activity, and production of bacteriocins[53]. Interestingly, the result of this study has shown that *Lactobacillus* was negatively correlated with laying hens' egg production and ADFI, which may be related to *Lactobacillus* deconjugating bile acids, affecting lipid metabolism and energy utilization[54,55].

Conclusions

In conclusion, dietary supplementation of OEO at 200 mg/kg and cinnamaldehyde at 20 mg/kg improved the egg production performance and the contents of MUFA, and decreased feed conversion ratio of late-phase laying hens. These beneficial effects of EO might be attributed to the selective modulations of cecum microbial communities. These findings may provide useful information for developing an effective and safe alternative to AGP in poultry industry.

Abbreviations

EO: essential oil; AGP: antibiotic growth promoter; OEO: oregano essential oil; FCR: feed conversion ratio; ADFI: average daily feed intake; SFA: saturated fatty acid; UFA: unsaturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; EFA: essential fatty acids

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Authors' contributions

The experiment was designed by Fei Gao, and conducted by Fei Gao. Experimental data were collected and analyzed by Fei Gao and Lianhua Zhang. The manuscript was written by Fei Gao and Lianhua Zhang, and revised by Hui Li, Fei Xia, Hongtong Bai, Zhiying Sun, Hongxia Cui and Lei

260 Shi. All authors have read and approved the final manuscript.

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264 **Availability of data and materials**

265 All data generated or analyzed during this study are included in this published article.

266 **Declarations**

267 **Ethics approval and consent to participate**

268 Animal management and experimental procedures were approved by the Shandong University of
269 Traditional Chinese Medicine.

270 **Consent for publication**

271 Not applicable.

272 **Competing interests**

273 There are no conflicts to declare.

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448 Table 1 Ingredient and nutrient levels of the experimental diets (% , as-fed basis)

Ingredients,		Nutrient levels ²	
Wheat	72.00	Metabolizable energy, MJ/kg	10.96
Soybean meal	14.50	Crude protein	16.06
Soybean oil	1.00	Calcium	3.86
Limestone	10.30	Available phosphorus	0.32
Premix ¹	2.20	L-Lysine	0.66
Total	100.00	DL-Methionine	0.37

449 ¹ Premix provided the following per kg of the diet: vitamin A, 443,040 IU; vitamin D₃, 99,968 IU;
 450 vitamin E, 1,136 IU; vitamin K₃ 113.6 mg; vitamin B₁ 113.6 mg; vitamin B₂, 284.0 mg; vitamin B₆,
 451 170.4 mg; vitamin B₁₂, 0.9 mg; D-biotin, 13.6 mg; D-pantothenic acid, 511.2 mg; nicotinamide,
 452 1818 mg; Choline chloride, 13,638 mg; Fe, 3,636 mg; Cu, 681.0 mg; Mn, 5454 mg; Zn, 4,090 mg;
 453 L-Lysine, 7,000 mg; DL-Methionine, 60,000 mg; NaCl, 3,000 mg

454 ² Calculated values

455

456

Table 2 Effects of dietary supplementation with essential oil on laying performance of laying hens¹

Items	Treatments ²					<i>P</i> -value
	Control	AGP	EO1	EO2	EO3	
Egg production						
Weeks 1-4	0.90 ± 0.03 ^a	0.91 ± 0.03 ^a	0.85 ± 0.02 ^b	0.91 ± 0.03 ^a	0.86 ± 0.02 ^b	0.001
Weeks 5-8	0.87 ± 0.03 ^b	0.92 ± 0.03 ^a	0.85 ± 0.03 ^b	0.92 ± 0.03 ^a	0.86 ± 0.02 ^b	0.001
Weeks 1-8	0.88 ± 0.01 ^b	0.91 ± 0.01 ^a	0.85 ± 0.01 ^c	0.91 ± 0.01 ^a	0.86 ± 0.01 ^c	0.001
Average egg weight, g						
Weeks 1-4	58.68 ± 0.57 ^b	59.46 ± 0.66 ^a	59.62 ± 0.75 ^a	58.97 ± 0.71 ^b	59.47 ± 0.71 ^a	0.001
Weeks 5-8	58.98 ± 0.48 ^c	59.65 ± 0.48 ^{ab}	59.96 ± 0.50 ^a	59.34 ± 0.50 ^b	59.93 ± 1.09 ^a	0.001
Weeks 1-8	58.81 ± 0.54 ^c	59.56 ± 0.58 ^a	59.79 ± 0.65 ^a	59.18 ± 0.64 ^b	59.70 ± 0.94 ^a	0.001
ADFI, g/hen per day						
Weeks 1-4	115.34 ± 4.82	120.34 ± 5.74	115.96 ± 5.44	116.93 ± 5.18	118.89 ± 4.91	0.649
Weeks 5-8	109.61 ± 3.17	116.34 ± 4.59	109.63 ± 3.32	111.03 ± 8.88	114.81 ± 5.91	0.389
Weeks 1-8	112.47 ± 4.86	118.32 ± 5.25	112.79 ± 5.37	113.98 ± 7.43	116.85 ± 5.48	0.193
FCR, g/g						
Weeks 1-4	2.18 ± 0.08	2.22 ± 0.07	2.28 ± 0.09	2.16 ± 0.07	2.30 ± 0.09	0.117
Weeks 5-8	2.25 ± 0.14	2.24 ± 0.14	2.27 ± 0.10	2.15 ± 0.04	2.32 ± 0.11	0.349
Weeks 1-8	2.22 ± 0.11 ^{ab}	2.23 ± 0.10 ^{ab}	2.28 ± 0.09 ^a	2.16 ± 0.06 ^b	2.31 ± 0.10 ^a	0.026
Broken egg production						
Weeks 1-4	1.57 ± 0.21	3.20 ± 1.23	3.61 ± 0.79	1.78 ± 0.99	2.21 ± 1.71	0.076
Weeks 5-8	3.60 ± 1.54	4.91 ± 1.33	3.09 ± 0.24	3.34 ± 1.14	5.21 ± 1.28	0.082
Weeks 1-8	2.58 ± 1.49	4.05 ± 1.50	3.35 ± 0.60	2.60 ± 13.32	3.72 ± 2.13	0.203

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¹ n = 5 replicates per treatment

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² Control, a basal diet; AGP, a basal diet + 5 mg/kg flavomycin; EO1, a basal diet + 100 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde; EO2, a basal diet +

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200 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde; and EO3, a basal diet + 300 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde

460 ^{a-c} Values within a row with no common superscripts differ significantly ($P < 0.05$)

461

462 Table 3 Effects of dietary supplementation with essential oil on egg quality of laying hens¹

Items	Treatments ²					<i>P</i> -value
	Control	AGP	EO1	EO2	EO3	
Eggshell thickness, 10 ⁻² mm						
Week 4	28.78 ± 0.90 ^a	28.56 ± 0.85 ^a	28.24 ± 0.48 ^{ab}	27.91 ± 0.83 ^b	27.87 ± 0.68 ^b	0.005
Week 8	28.73 ± 0.97	28.89 ± 1.53	29.02 ± 1.29	29.42 ± 1.30	29.20 ± 1.38	0.643
Eggshell strength, kg						
Week 4	3.55 ± 0.63	3.45 ± 0.66	3.45 ± 0.75	3.46 ± 0.68	3.57 ± 0.54	0.971
Week 8	3.42 ± 0.76	3.41 ± 0.96	3.45 ± 0.51	3.61 ± 0.63	3.55 ± 0.59	0.918
Shape index						
Week 4	1.35 ± 0.05	1.34 ± 0.03	1.34 ± 0.04	1.36 ± 0.05	1.38 ± 0.05	0.080
Week 8	1.34 ± 0.07	1.35 ± 0.06	1.34 ± 0.06	1.34 ± 0.03	1.37 ± 0.04	0.160
Albumen height, mm						
Week 4	6.53 ± 1.36	6.44 ± 1.47	6.14 ± 0.81	6.40 ± 1.16	5.76 ± 1.02	0.393
Week 8	5.99 ± 1.51	6.05 ± 1.24	5.98 ± 1.07	5.96 ± 0.74	5.90 ± 1.35	0.998
Haugh unit						
Week 4	79.53 ± 10.70	78.96 ± 13.26	77.83 ± 5.61	78.77 ± 8.50	73.65 ± 8.24	0.462
Week 8	75.70 ± 13.72	75.81 ± 10.30	76.21 ± 7.84	76.70 ± 5.83	74.28 ± 10.59	0.974

463 ¹ n = 5 replicates per treatment

464 ² Control, a basal diet; AGP, a basal diet + 5 mg/kg flavomycin; EO1, a basal diet + 100 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde; EO2, a basal diet +
 465 200 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde; and EO3, a basal diet + 300 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde

466 ^{a-c} Values within a row with no common superscripts differ significantly ($P < 0.05$)

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Table 4 Effects of dietary supplementation with essential oil on fatty acid composition of egg yolk.¹ (g/100g)

Fatty acid	Treatments ²					P-value
	Control	AGP	EO1	EO2	EO3	
C4:0	0.002 ± 0.001 ^b	0.003 ± 0.001 ^b	0.005 ± 0.002 ^b	0.005 ± 0.002 ^b	0.008 ± 0.003 ^a	0.005
C10:0	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.907
C12:0	0.005 ± 0.000 ^b	0.006 ± 0.000 ^a	0.006 ± 0.000 ^a	0.006 ± 0.000 ^a	0.006 ± 0.000 ^a	0.003
C14:0	0.277 ± 0.015	0.273 ± 0.008	0.269 ± 0.0182	0.267 ± 0.014	0.271 ± 0.015	0.814
C15:0	0.044 ± 0.002 ^a	0.043 ± 0.003 ^a	0.041 ± 0.006 ^{ab}	0.037 ± 0.005 ^{bc}	0.034 ± 0.004 ^c	0.006
C16:0	18.656 ± 0.164	18.581 ± 1.582	19.411 ± 0.527	19.050 ± 0.593	19.220 ± 0.825	0.520
C18:0	6.136 ± 0.199 ^b	6.565 ± 0.221 ^a	6.748 ± 0.212 ^a	6.499 ± 0.296 ^b	6.624 ± 0.400 ^a	0.026
C21:0	0.047 ± 0.004	0.057 ± 0.004	0.060 ± 0.004	0.059 ± 0.011	0.057 ± 0.007	0.056
C24:0	0.097 ± 0.013	0.092 ± 0.007	0.091 ± 0.009	0.090 ± 0.011	0.098 ± 0.016	0.716
C16:1	2.660 ± 0.368	2.818 ± 0.187	2.664 ± 0.326	2.799 ± 0.379	2.753 ± 0.121	0.866
C18:1, cis(n-9)	15.191 ± 0.728 ^c	15.245 ± 0.181 ^{bc}	15.490 ± 0.496 ^{bc}	16.069 ± 0.754 ^{ab}	16.452 ± 0.647 ^a	0.012
C20:1 n9	0.131 ± 0.020 ^b	0.148 ± 0.007 ^{ab}	0.156 ± 0.015 ^a	0.158 ± 0.011 ^a	0.153 ± 0.009 ^a	0.034
C18:2, cis(n-6)	9.518 ± 0.296	9.505 ± 0.667	9.336 ± 0.941	9.157 ± 0.695	9.573 ± 0.939	0.899
C18:3 n-3	0.360 ± 0.017	0.368 ± 0.050	0.361 ± 0.042	0.333 ± 0.043	0.349 ± 0.042	0.675
C20:2	0.138 ± 0.006	0.153 ± 0.008	0.159 ± 0.013	0.148 ± 0.017	0.142 ± 0.008	0.059
C20:3 n-6	0.149 ± 0.008	0.154 ± 0.012	0.155 ± 0.009	0.143 ± 0.007	0.139 ± 0.010	0.057
C20:4 n-6	1.397 ± 0.038	1.430 ± 0.042	1.420 ± 0.091	1.382 ± 0.124	1.460 ± 0.068	0.578
C20:5	0.010 ± 0.002 ^b	0.013 ± 0.001 ^a	0.014 ± 0.000 ^a	0.013 ± 0.001 ^a	0.014 ± 0.001 ^a	0.001
C22:6	0.701 ± 0.060	0.731 ± 0.023	0.713 ± 0.047	0.715 ± 0.081	0.780 ± 0.071	0.292
SFA	25.392 ± 0.304	26.309 ± 1.556	26.778 ± 0.368	26.013 ± 0.622	26.319 ± 1.203	0.204
UFA	30.260 ± 0.197	30.565 ± 0.181	30.468 ± 0.899	30.917 ± 1.028	31.816 ± 1.166	0.061
MUFA	17.983 ± 0.468 ^c	18.212 ± 0.261 ^c	18.311 ± 0.719 ^{bc}	19.0256 ± 0.438 ^{ab}	19.358 ± 0.726 ^a	0.003
PUFA	12.276 ± 0.299	12.355 ± 0.732	12.158 ± 1.103	11.891 ± 0.833	12.457 ± 1.128	0.867

n-3 PUFA	1.062 ± 0.071	1.100 ± 0.067	1.074 ± 0.081	1.048 ± 0.101	1.129 ± 0.114	0.627
n-6 PUFA	11.064 ± 0.292	11.089 ± 0.669	10.911 ± 1.041	10.682 ± 0.742	11.172 ± 1.011	0.876
n-3/n-6	0.096 ± 0.007	0.099 ± 0.003	0.099 ± 0.003	0.098 ± 0.007	0.101 ± 0.002	0.605
UFA/SFA	1.197 ± 0.001	1.196 ± 0.067	1.144 ± 0.039	1.189 ± 0.051	1.210 ± 0.036	0.221
EFA	11.276 ± 0.295	11.303 ± 0.712	11.117 ± 1.053	10.872 ± 0.780	11.383 ± 1.047	0.874

¹ n = 5 replicates per treatment

² Control, a basal diet; AGP, a basal diet + 5 mg/kg flavomycin; EO1, a basal diet + 100 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde; EO2, a basal diet + 200 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde; and EO3, a basal diet + 300 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde

SFA, saturated fatty acid; UFA, unsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; EFA, essential fatty acids; n-3 PUFA= C18:3 n-3 + C22:6; n-6 PUFA= C18:2, cis(n-6) + C20:3 n-6+ C20:4 n-6

^{a-c} Values within a row with no common superscripts differ significantly ($P < 0.05$)

Table 6 Effect of dietary supplementation with essential oil on alpha diversity in cecum¹

Items	Treatments ²			<i>P</i> -value
	Control	AGP	EO2	
Shannon	7.94 ± 0.26	7.84 ± 0.83	8.18 ± 0.19	0.579
Simpson	0.99 ± 0.003	0.98 ± 0.014	0.99 ± 0.004	0.627
Chao1	855.41 ± 122.10 ^{ab}	790.28 ± 157.48 ^b	1006.06 ± 87.40 ^a	0.050
ACE	850.20 ± 123.40	786.00 ± 155.13	996.60 ± 86.30	0.055

¹ n = 5 replicates per treatment

² Control, a basal diet; AGP, a basal diet + 5 mg/kg flavomycin; EO2, a basal diet + 200 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde;

^{a-b} Values within a row with no common superscripts differ significantly (*P* < 0.05)

Table 7 Differences of bacterial distribution in cecal digesta between the control, AGP and EO2 groups ¹

Items, %	Control ²	AGP	EO2	<i>P</i> -value
Phyla				
Desulfobacterota	3.72 ± 0.79 ^a	2.04 ± 0.63 ^b	2.66 ± 0.71 ^b	0.010
Actinobacteriota	1.34 ± 1.04 ^b	1.51 ± 0.55 ^b	3.88 ± 1.91 ^a	0.016
Class				
Desulfovibrionia	3.69 ± 0.75 ^a	2.02 ± 0.64 ^b	2.61 ± 0.67 ^b	0.008
Family				
Lachnospiraceae	6.79 ± 1.54 ^b	10.16 ± 1.08 ^a	6.69 ± 1.22 ^b	0.002
Desulfovibrionaceae	3.69 ± 0.76 ^a	2.02 ± 0.64 ^b	2.61 ± 0.68 ^b	0.008
Genus				
<i>Desulfovibrio</i>	3.63 ± 0.77 ^a	2.00 ± 0.63 ^b	2.56 ± 0.69 ^b	0.010

¹ n = 5 replicates per treatment

² Control, a basal diet; AGP, a basal diet + 5 mg/kg flavomycin; EO2, a basal diet + 200 mg/kg oregano essential oil + 20 mg/kg cinnamaldehyde;

^{a-b} Values within a row with no common superscripts differ significantly (*P* < 0.05)

A

B

Fig. 1. Venn diagram of ASV (A) and PLS-DA (B) of cecum microbiota in the three groups. Control, hens received a basal diet; AGP, hens received a basal diet supplemented with 5 mg/kg flavomycin, EO2, hens received a basal diet supplemented with 200 mg/kg oregano essential oil and 20 mg/kg cinnamaldehyde.

Fig.2. Microbial composition in the cecum of laying hens at phylum level (A), class level (B) family level (C) and genus level (D). Control, hens received a basal diet; ACP, hens received a basal diet supplemented with 5 mg/kg flavomycin, EO2, hens received a basal diet supplemented with 200 mg/kg oregano essential oil and 20 mg/kg cinnamaldehyde.

Correlation Network

Fig.4. Pearson's correlation analysis between the abundances of cecal microbiota and production at genus level. FCR, feed conversion ratio; ADFI, average daily feed intake