Montmorillonite combined organic acid or essential oil complex improves the intestinal health status of laying hens

Jifa Chen (jifachen66@jxycu.edu.cn)
Yichun University
Xiaofeng Geng
Yichun University
Ali Seif Ali
Yichun University

Research

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Abstract

Background: This study was conducted to evaluate the effects of montmorillonite combined organic acid (MOA) complex and montmorillonite combined essential oil (MEO) complex on the laying performance and intestinal barrier functions in laying hens.

Methods: Two hundred and seventy 29-wk-old laying hens were randomly assigned to 3 treatments consisted of 6 replicates of 15 hens, receiving the following diets for 10 wks, respectively: 1) basal diet; 2) the basal diet added with 0.65 g/kg of MOA complex; 3) the basal diet added with 0.70 g/kg of MEO complex.

Results: Dietary supplementation with both MOA and MEO complexes tended to increase ($P = 0.094$) the hens' egg production and improved numerically ($P > 0.05$) the egg mass and feed conversion ratio during 6–10 weeks of the experiment. Dietary MOA and MEO complexes decreased ($P < 0.05$) the relative amount of *Clostridium perfringens* and the mRNA expression levels of myeloid differentiation factor 88 (*MyD88*) and interleukin-1 beta (*IL-1β*) in the ileum while increased ($P < 0.05$) the activity of suctase and villus height-to-crypt depth ratio of the ileum. Besides, dietary inclusion of MEO complex increased ($P < 0.05$) the mRNA expression level of claudin-5 in the ileum. The MOA complex supplementation reduced ($P < 0.05$) the relative amount of *Salmonella* and nuclear factor kappa B P65 (*NF-κB p65*) mRNA expression level of the ileum but enhanced ($P < 0.05$) the alkaline phosphatase activity in the ileum and villus height-to-crypt depth ratio of the jejunum.

Conclusions: Dietary both MOA and MEO complexes could improve the intestinal health status of laying hens, which may contribute to the partially increase in hens' laying performance.

Background

The intestine not only plays an important role in digesting and absorbing nutrients but also serves as an innate barrier to maintain the internal homeostasis of the body [1]. The healthy intestine is important for intestinal microflora, gut immunity, and digestion and absorption of nutrients, contributing to better growth, higher productivity, and successful feed efficiency. However, there are growing concerns about poultry intestinal health problems with the ban of antibiotic growth promoters as feed additives, the deteriorating farm environment, and the continuous improvement of productivity in poultry. Concerns on higher performance and food and environmental safety have driven researchers to exploit new efficient feed additives, giving power to maintain the gut health of poultry such as clays, probiotics, plant extracts, and feed enzymes.

Montmorillonite (MMT), an aluminosilicate mineral clay, has been utilized to prevent and treat diarrhea in human for a long time and it has also been used as an enterosorbent to improve the animal's performance [2–4]. MMT could increase performance through binding mycotoxins and bacterial toxins in the intestinal tract, regulating the balance of intestinal microbiota, improving the activity of digestive enzyme, and maintaining the gut barrier functions [5–7]. And MMT has also been found to be one of the most promising intestinal health regulators [3, 4, 8]. Organic acids (OA) and essential oils (EO) have also attracted increased attention from the poultry industry. In generally, OA and EO modify the intestinal microbiota, improve the gut morphology, exert antioxidant property, enhance the animal's immune function, and increase digestion and absorption of nutrients [9–12]. These properties help to understand the OA and EO as feed additives for promoting growth or production performance in poultry.

In recent years, it has been reported that the combination of additives performs better than a single additive in improving the gut health status and animal's performance. Our previous study indicates that dietary

supplementation with an MMT and *Bacillus subtilis* mixture improve the intestinal barrier functions of laying hens, resulting in better laying performance [4]. Dietary OA and EO compounds could reduce intestinal harmful bacteria, enhance digestive enzyme activity, and contribute to beneficial effects on feed efficiency of broilers [10]. A blend of MMT and OA is effective on control the necrotic enteritis caused by *Eimeria maxima* and *Clostridium perfringens* in broiler chickens [13]. However, supplementation of the combination of MMT and OA or EO in diets for laying hens has not been reported yet. We hypothesized that the two complexes may have synergistic effects on improving the hens' intestinal health status and better laying performance. Therefore, an experiment was carried out to evaluate the effects of MMT combined OA (MOA) complex and MMT combined EO (MEO) complex on performance, gut microflora, intestinal brush border enzyme activity and barrier functions in laying hens.

Methods

The experiment was conducted under approval of the experimental protocol by Institutional Animal Care and Use Committee of Yichun University.

Materials

Both MOA and MEO complexes were commercial products. The main components of MOA complex were as follow: MMT 80% and OA (main ingredients included formic acid, propionate acid, and butyric acid) > 10%. And the main components of MEO complex were: MMT 75% and EO (main ingredients included eugenol and cinnamaldehyde) > 4%.

Experimental design, diets, and management

Two hundred and seventy 29-wk-old Lohmann Brown laying hens were randomly allocated to 3 treatments with 6 replicates per treatment and 15 hens per replicate (5 adjacent cages, 3 hens per cage) after 1 wk adaptation period to the stainless steel cages (40 cm × 40 cm × 35 cm). Hens in the 3 treatments were fed the following diets for 10 wks, respectively: 1) basal diet (CON group); 2) the basal diet added with 0.65 g/kg of MOA complex (MOA group); 3) the basal diet added with 0.70 g/kg of MEO complex (MEO group).

The basal diet was a corn-soybean meal based and formulated in accordance with the established guidelines [14] to meet the nutrient requirements of laying hens. The ingredients and nutrient amounts of the basal diet were shown in Table 1. Hens were housed in an environmentally controlled room. During 1–10 weeks of the experiment, the average temperature and relative humidity in the room were 25.55 °C and 77.23%, respectively. Hens were allowed *ad libitum* access to feed and water throughout the experiment and were exposed to a 16:8 light: dark cycle.

Table 1
Formulation and calculated composition of the basal diet (air-dry basis)

Items (%, unless otherwise indicated)	Content
Ingredients	
Corn	64.00
Soybean meal	24.00
Limestone	8.00
Dicalcium phosphate	1.00
Premix[1]	3.00
Total	100.00
Nutrient composition (calculated)	
Metabolisable energy (MJ/kg)	11.25
Crude protein	16.60
Calcium	3.51
Available phosphorus	0.34
Lysine	0.87
Methionine + cystine	0.65

[1]The premix provided per kilogram of diet: vitamin A 7,500 IU, vitamin D3 3,000 IU, vitamin E 20 IU, vitamin K3 2 mg, vitamin B1 1.98 mg, vitamin B2 4.98 mg, vitamin B6 4.98 mg, vitamin B12 0.02 mg, *DL*-methionine 0.98 g, choline chloride 400 mg, nicotinic acid 30 mg, pantothenate 15 mg, folic acid 0.78 mg, biotin 0.2 mg, Fe (from ferrous sulfate) 75 mg, Cu (from copper sulfate) 10 mg, Se (from sodium selenite) 0.3 mg, Zn (from zinc sulfate) 70 mg, Mn (from manganese sulfate) 60 mg, I (from potassium iodide) 1 mg, Ca 3.3 g, P 1.05 g, NaCl 3.5 g.

Sample collection

At the end of the experiment, 6 hens per treatment (1 hen/replicate) were randomly selected and euthanized by cervical dislocation and necropsied immediately [4]. The jejunum (from the end of pancreatic loop to Meckel's diverticulum) and ileum (from Meckel's diverticulum to the ileocecal junction) were then isolated and rapidly excised. Approximately 2-cm segments of the medial parts of the jejunum and ileum were removed and flushed with ice-cold PBS at pH 7.4 to remove the contents and immediately fixed in 10% formalin solution for intestinal morphology measurement. The fresh ileal digesta samples were collected later and store at − 80 °C until analysis for microflora amount. After that, the remaining ileal tract was cut open lengthways, and cleaned thoroughly with ice-cold PBS, and the ileal mucosa was scraped carefully by an aseptic glass slide and then packed in sterile aluminum foil, and stored at − 80 °C until the determination of brush border enzyme activity and gene expression.

Performance

During the experimental period, egg production and egg weight were recorded daily by replicate, and feed consumption was obtained weekly by replicate to calculate egg production, egg mass, and feed conversion ratio (FCR).

Ileal microflora

Bacterial DNA was isolated using the Stool DNA Kit (DP328, Tiangen, Tiangen Biotech Co., Ltd., Beijing, China) from the snap-frozen ileal digesta according to the manufacturer's instructions. DNA integrity was checked on 0.8% agarose gel with ethidium bromide staining. And DNA concentration and purity were determined from OD260/280 readings using a NanoDrop ND-2000 UV spectrophotometer (Thermo Fisher Scientific, Waltham). After that, all the qualified DNA samples were diluted in diethyl polycarbonate treated water to appropriate concentration (100 ng/μL). The primer sequences for the target and reference bacteria (*Bifidobacterium*, *Lactobacillus_acidophilus*, *C. perfringens*, *Escherichia coli*, *Salmonella*, *Enterococcus*, and total bacteria) are listed in Table 2 as previously published by Chen et al. [15].

Table 2

Primers used for quantitative real-time PCR[1]

Items	Accession number or reference	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
Beta-actin	NM_205518.1	GAGAAATTGTGCGTGACATCA	CCTGAACCTCTCATTGCCA
Claudin-1	NM_001013611	TGGAGGATGACCAGGTGAAGA	GAGCCACTCTGTTGCCATA
Claudin-5	NM_204201	CATCACTTCTCCTTCGTCAGC	GCACAAAGATCTCCCAGGTC
ZO-1	XM_413773.4	CTTCAGGTGTTTCTCTTCCTCCTC	CTGTGGTTTCATGGCTGGATC
Occludin	NM_205128.1	TCATCGCCTCCATCGTCTAC	TCTTACTGCGCGTCTTCTGG
Mucin-2	XM_421035.2	TTCATGATGCCTGCTCTTGTG	CCTGAGCCTTGGTACATTCTTGT
Mucin-5ac	XM_003641322.2	TGTGGTTGCTATGAGAATGGA	TTGCCATGGTTTGTGCAT
TLR2	NM_204278.1	CCTGCAACGGTCATCTCAG	GTCTCAGGGCTTGTTCTTCAG
TLR4	NM_001030693	CTGACCTACCCATCGGACAC	GCCTGAGAGAGGTCAGGTTG
MyD88	NM_001030962	AGAAGGTGTCGGAGGATGGTG	GGGCTCCAAATGCTGACTGC
NF-κB p65	NM_205129	GTGTGAAGAAACGGGAACTG	GGCACGGTTGTCATAGATGG
IL-1β	NM_204524.1	ACTGGGCATCAAGGGCTA	GGTAGAAGATGAAGCGGGTC
TNF-α	AY765397.1	CTCCGCAGTACTCAGGACAGC	TCAGAGCATCAACGCAAAAGG
Total bacteria	Chen et al., (2019a)	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC
Escherichia coli	HF936924.1	GTGTAGCGGTGAAATGCGTAG	TCAAGGGCACAACCTCCAAG
Salmonella	AF332600.1	GAGTGGCGGACGGGTGAGTA	TGGGCACATCTATGGCAAG
Bifidobacterium	AB697147.1	GCAACGCGAAGAACCTTACCT	CTTGACCCAACATCACGACA
Lactobacillus	AB680529.1	AGCGAACAGGATTAGATACCC	GATGGCACTAGATGTCAAGACC
Enterococcus	KC699233.1	CCGTAAACGATGAGTGCTAAG	AAGGATGTCAAGACCTGGTAAG
Clostridium perfringens	Chen et al. (2019a)	AAAGATGGCATCATCATTCAAC	TACCGTCATTATCTTCCCCAAA

[1] *ZO-1* zonula occludens-1; *TLR2* toll-like receptor 2; *TLR4* toll-like receptor 4; *MyD88* myeloid differentiation factor 88; *NF-κB P65* nuclear factor kappa B P65; *IL-1β* interleukin 1 beta; *TNF-a* tumor necrosis factor alpha.

Real-time PCR was carried out on a Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA) following optimized PCR protocols. In brief, the reaction mixture was prepared using 10 μl of SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd., Dalian, China), 2 μl of DNA, 0.8 μl of forward primer, 0.8 μl of reverse primer, and 6.4 μl of double-distilled water. Each sample was performed in triplicate. Optimized cycling conditions of all bacterial were 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing and extension at 60°C for 40 s. The relative amount of bacterial was calculated using the method $2^{-\Delta\Delta Ct}$ after normalization against

the reference total bacteria as described by Chen et al. [15] and Feng et al. [16]. And the values of the CON group were used as a calibrator.

Intestinal morphology

Intestinal tissue samples were dehydrated, cleared, and embedded in paraffin. Serial sections were then cut at 5-um thickness, deparaffinized in xylene, rehydrated, and stained with hematoxylin and eosin as per standard paraffin embedding procedures for examination using a microscope. Villus height and crypt depth of 10 well-oriented villi per segment were measured using an image processing and analyzing system (Olympus IX51 inverted microscope, Olympus Optical Co., Ltd., Tokyo, Japan). The villus height-to-crypt depth ratio was subsequently calculated.

Brush border enzyme activity

Approximately 0.5 g of ileal mucosa sample was used to prepare mucosal homogenate. The mucosa samples were first diluted in the ratio of 1:9 (wt/vol) with ice-cold physiological saline solution, and homogenized using an Ultra-Turrax homogenizer (T10BS25, IKA, Baden-Wurttemberg, Germany) in an ice bath for 20 s, and then centrifuged at 2,000 × *g* at 4℃ for 10 min. The supernatant was collected and used for analysis. The concentrations of protein in the mucosal homogenate were quantified by using an assay kit (A045-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activities of alkaline phosphatase (AKP), suctase, maltase, and lactase in the supernatant were determined using the corresponding assay kits (A059-2-2, A082-2-1, A082-3-1, A082-1-1, respectively, Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) and a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, CT) following the manufacturer's protocol.

Ileal mucosa gene expression

Total RNA from ileal tissues was extracted with Trizol reagent (Invitrogen Life Technologies, Carlsbad, USA). The concentration and quality of the isolated RNA were measured by spectrophotometer (NanoDrop ND-2000 UV, Thermo Fisher Scientific, Waltham) and agarose gel electrophoresis, respectively. After that, reverse transcription was conducted by a reverse transcription kits (Takara Biotechnology Co., Ltd., Dalian, China) in which the reaction system (20 μl) included 1 μg of total RNA. The cDNA was amplified in a 10 μl quantitative real-time PCR system using SYBR Premix Ex Taq II kit (Takara Biotechnology Co., Ltd., Dalian, China). The real-time PCR system contained SYBR Premix Ex Taq II (2 ×), diluent cDNA, and 0.2 μmol/l each primer for the target gene.

The real-time PCR quantitative assay was conducted on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The amplification consisted of initial incubation for 30 s at 95℃, 40 cycles of denaturation at 95℃ for 5 s, annealing and extension at 60℃ for 40 s. The primer pairs for beta-actin and target genes are given in Table 2. Experiments on each sample were analyzed in triplicate. The mRNA relative expression was calculated as per the method of Livak and Schmittgen [17].

Statistical analysis

All data were subjected to one-way ANOVA analysis by using the SAS statistical software (Version 9.2, SAS Institute, Cary, NC, USA), and the replicate was used as the experimental unit. When significant differences were found ($P < 0.05$), a Tukey's test was performed. Data are presented as means with their pooled standard errors. In all analyses, $P < 0.05$ was considered significantly different, and $0.05 \leq P < 0.10$ was considered a tendency. Before analysis, the egg production was subjected to an arcsine transformation.

Results

Performance

There were no significant differences in egg production, egg mass, or FCR among all treatments, but the MOA complex and MEO complex supplementation tended to increase ($P = 0.094$) the egg production by 2.79% and 2.35%, respectively. Dietary both MOA and MEO complexes also improved numerically ($P > 0.05$) the egg mass and FCR during 6–10 weeks of the experiment (Table 3).

Table 3

Effects of MOA complex and MEO complex on the performance in laying hens[1,2]

Items	Treatments			Pooled SEM	*P*-value
	CON	MOA	MEO		
1–5 weeks of the experiment					
Egg production (%)	95.12	95.49	95.50	0.37	0.851
Egg mass (g/day per hen)	56.72	57.32	57.58	0.39	0.676
FCR (g of feed/g of egg)	2.05	2.03	2.00	0.01	0.262
6–10 weeks of the experiment					
Egg production (%)	93.53	96.14	95.73	0.55	0.094
Egg mass (g/day per hen)	57.28	58.19	58.93	0.39	0.233
FCR (g of feed/g of egg)	2.14	2.12	2.09	0.01	0.370
1–10 weeks of the experiment					
Egg production (%)	94.33	95.81	95.62	0.39	0.251
Egg mass (g/day per hen)	57.00	57.75	58.26	0.35	0.363
FCR (g of feed/g of egg)	2.09	2.07	2.04	0.01	0.264

[1]n = 6 per treatment group. Dietary treatments were as follows: *CON* basal diet; *MOA* basal diet + 0.65 g/kg of montmorillonite combined organic acid (MOA) complex; *MEO* basal diet + 0.70 g/kg of montmorillonite combined essential oil (MEO) complex.

[2]*FCR* feed conversion ratio.

Ileal microflora

The relative amounts of *Bifidobacterium*, *Lactobacillus_acidophilus*, *E. coli*, and *Enterococcus* between MOA, MEO, and CON group indicated no statistically significant difference ($P > 0.05$; Table 4). However, dietary supplementation with both MOA and MEO complexes decreased ($P < 0.05$) the relative amount of *C. perfringens*. The relative amount of *Salmonella* in MEO group significantly reduced ($P < 0.05$) compared with CON and MOA group.

Table 4

Effects of MOA complex and MEO complex on relative amounts of ileal microflora in laying hens[1,2]

Items	Treatments			Pooled SEM	*P*-value
	CON	MOA	MEO		
Bifidobacterium	1.00	2.37	1.44	0.45	0.502
Lactobacillus_acidophilus	1.00	1.70	1.81	0.18	0.146
Clostridium perfringens	1.00[a]	0.45[b]	0.16[c]	0.10	< 0.001
Escherichia coli	1.00	0.90	1.06	0.26	0.976
Salmonella	1.00[a]	1.04[a]	0.36[b]	0.11	0.010
Enterococcus	1.00	1.49	1.35	0.10	0.101

[a–c]Means within a row with different superscripts differ significantly ($P < 0.05$).

[1]n = 6 per treatment group. Dietary treatments were as follows: *CON* basal diet; *MOA* basal diet + 0.65 g/kg of montmorillonite combined organic acid (MOA) complex; *MEO* basal diet + 0.70 g/kg of montmorillonite combined essential oil (MEO) complex.

[2]Expressed in arbitrary units. The relative amount of each target bacteria for the CON group fed with the basal diet was assigned a value of 1 and normalized against total bacteria.

Intestinal morphology

Compared with CON group, the villus height-to-crypt depth ratio of the jejunum in MEO group significantly increased ($P < 0.05$; Table 5). The crypt depth of the jejunum in MEO group was higher ($P < 0.05$) than that in MOA group. As expected, with the MOA and MEO complexes supplementation, the villus height-to-crypt depth ratio of the ileum significantly increased ($P < 0.05$).

Table 5
Effects of MOA complex and MEO complex on intestinal morphology in laying hens

Items	Treatments			Pooled SEM	*P*-value
	CON	MOA	MEO		
Jejunum					
Villus height (μm)	739.47	919.10	721.70	46.11	0.156
Crypt depth (μm)	169.79^{ab}	196.97^{a}	126.62^{b}	10.70	0.015
Villus height: crypt depth	4.39^{b}	4.65^{b}	5.89^{a}	0.28	0.037
Ileum					
Villus height (μm)	469.25	461.87	520.42	29.41	0.702
Crypt depth (μm)	150.50	106.98	116.08	8.56	0.088
Villus height: crypt depth	3.20^{b}	4.29^{a}	4.52^{a}	0.16	< 0.001

$^{a–b}$ Means within a row with different superscripts differ significantly ($P < 0.05$).

[1]n = 6 per treatment group. Dietary treatments were as follows: *CON* basal diet; *MOA* basal diet + 0.65 g/kg of montmorillonite combined organic acid (MOA) complex; *MEO* basal diet + 0.70 g/kg of montmorillonite combined essential oil (MEO) complex.

Brush border enzyme activity

As indicated in Table 6, the activity of suctase in the ileal mucosa significantly increased ($P < 0.05$) in both MOA and MEO groups compared with CON group. The AKP activity of the ileum in MEO group significantly enhanced ($P < 0.05$) compared with CON group. There were no significant differences ($P > 0.05$) in the activities of maltase and lactase in the ileal mucosa among all groups.

Table 6
Effects of MOA complex and MEO complex on the activities of brush border enzymes in the ileum of laying hens

Items	Treatments			Pooled SEM	*P*-value
	CON	MOA	MEO		
Suctase (U/mg of protein)	62.93[b]	117.11[a]	117.51[a]	10.79	0.049
Maltase (U/mg of protein)	157.32	93.01	212.48	21.79	0.073
Lactase (U/mg of protein)	127.46	240.44	212.48	35.65	0.441
Alkaline phosphatase (King unit/g of protein)	76.57[b]	120.88[ab]	141.11[a]	10.51	0.026

[a–b]Means within a row with different superscripts differ significantly ($P < 0.05$).

[1]n = 6 per treatment group. Dietary treatments were as follows: *CON* basal diet; *MOA* basal diet + 0.65 g/kg of montmorillonite combined organic acid (MOA) complex; *MEO* basal diet + 0.70 g/kg of montmorillonite combined essential oil (MEO) complex.

Ileal mucosa gene expression

As shown in Table 7, the mRNA expression levels of claudin-1, zonula occludens-1, mucin-2, mucin-5ac, toll-like receptor (TLR) 2, *TLR4*, or tumor necrosis factor alpha (*TNF-α*) were no affected ($P > 0.05$) by the MOA or MEO complex supplementation. Compared with CON group, the mRNA expression level of claudin-5 in MOA group significantly up-regulated ($P < 0.05$). The occludin mRNA expression level in MEO group significantly increased ($P < 0.05$) compared with MOA group. The mRNA expression levels of myeloid differentiation factor 88 (*MyD88*) and interleukin 1 beta (*IL-1β*) in both MEO and MOA groups significantly down-regulated ($P < 0.05$) compared with CON group. Besides, the nuclear factor kappa B (NF-κB) p65 mRNA expression level in MEO group was significantly lower ($P < 0.05$) than that in CON group.

Table 7

Effects of MOA complex and MEO complex on the ileal mucosa gene expression of laying hens[1,2,3]

Items	Treatments			Pooled SEM	*P*-value
	CON	MOA	MEO		
Claudin-1	1.00	1.25	0.93	0.13	0.593
Claudin-5	1.00^{b}	1.83^{a}	1.51^{ab}	0.14	0.025
Occludin	1.00^{ab}	0.71^{b}	1.73^{a}	0.18	0.042
ZO-1	1.00	0.39	1.00	0.12	0.056
Mucin-2	1.00	0.65	1.04	0.12	0.351
Mucin-5ac	1.00	0.62	1.24	0.18	0.370
TLR2	1.00	0.64	1.05	0.11	0.224
TLR4	1.00	0.68	0.83	0.10	0.437
MyD88	1.00^{a}	0.52^{b}	0.75^{b}	0.06	0.002
NF-κB p65	1.00^{a}	0.70^{ab}	0.60^{b}	0.07	0.049
IL-1β	1.00^{a}	0.48^{b}	0.63^{b}	0.07	0.003
TNF-α	1.00	0.77	1.11	0.07	0.089

$^{a-b}$ Means within a row with different superscripts differ significantly ($P < 0.05$).

[1]n = 6 per treatment group. Dietary treatments were as follows: *CON* basal diet; *MOA* basal diet + 0.65 g/kg of montmorillonite combined organic acid (MOA) complex; *MEO* basal diet + 0.70 g/kg of montmorillonite combined essential oil (MEO) complex.

[2] Expressed in arbitrary units. The mRNA level of each target gene for the CON group fed with the basal diet was assigned a value of 1 and normalized against beta-actin.

[3]*ZO-1* zonula occludens-1; *TLR2* toll-like receptor 2; *TLR4* toll-like receptor 4; *MyD88* myeloid differentiation factor 88; *NF-κB P65* nuclear factor kappa B P65; *IL-1β* interleukin 1 beta; *TNF-α* tumor necrosis factor alpha.

Discussion

In the present study, dietary supplementation with both MOA and MEO complexes tended to increase the egg production and improved numerically the egg mass and FCR during 6–10 weeks of the experiment. It is generally accepted that healthy intestine is important for better growth and higher production in poultry. The intestinal microorganisms can compete with the host for nutrients in the intestinal lumen [18]. And excessive breeding of the pathogenic bacteria in the intestinal tract will consume a large amount of nutrients, reduce the feed efficiency, and compromise the animal's performance finally. Thus, the reduction of intestinal harmful bacteria can be considered as a factor for promoting animal's growth or production. In this study, the decreased amounts of *C. perfringens* and *Salmonella*, which were observed in the ileal digesta of laying hens fed with MOA or MEO complex supplementation diets, indicated an improvement in intestinal microflora. This result was similar to the earlier

findings reported by Yang et al. [10], Chen et al. [15], and Adaszynska and Szczerbinska [19]. It is known that inhibition of the population of pathogenic bacteria in the gut is beneficial to maintain the intestinal barrier functions. It was speculated that dietary supplementation with MOA or MEO complex could partially improve the hens' laying performance through modifying the intestinal microflora to improve the gut barrier functions in the present study.

The family of TLR is capable of recognizing diverse pathogen-associated molecular patterns such as lipopeptides (main recognized by *TLR2*) and lipopolysaccharides (main recognized by *TLR4*) and initiating an inflammatory signaling cascade [20]. Stimulation of the extracellular domain of TLR triggers the intracellular association of *MyD88* with its cytosolic domain for the activation of NF-κB. Once activated, the *NF-κB p65* subunit is released after inhibitor of NF-κB (IκB) phosphorylation. Phosphorylated *NF-κB p65* then translocates into the nucleus to switch target gene transcription, including a large number of pro-inflammatory mediators and cytokines (e.g., *TNF-α*, *IL-1β*, and *IL-6*) [21]. In this study, the MOA and MEO complexes supplementation down-regulated the mRNA expression levels of *MyD88*, *NF-κB p65*, and *IL-1β* and reduced numerically the *TLR4* mRNA expression of the ileum, indicating that MOA and MEO complexes may inhibit the inflammatory responses by modulating the signal pathway of *TLR4*/*MyD88*/NF-κB. This finding was similar to the results of Chen et al. [8], Yang et al. [10], and Liu et al. [22]. Many harmful bacteria such as *E. coli* and *Salmonella* exhibit structural characteristics of the lipopolysaccharide-constructed cell membrane, so they can be recognized by *TLR4*, and they then transmit signals to activate inflammatory responses. Thus, we suggested that the suppression of proinflammatory cytokines might be attributed to the inhibition of the population of gut harmful bacteria and improvement of the intestinal micro-ecological environment. It is reported that cytokines play a critical role in the modulation of the intestinal epithelial tight junction barrier [23]. And the reduction of proinflammatory cytokines may contribute to the integrity of intestinal epithelial cells and tight junctions in the gut of laying hens.

A single layer of intestinal epithelial cells can be connected by junctional complexes such as tight junctions and gap junctions [24], which forms a physical barrier between the intestinal lumen [25]. Gross villus height and crypt depth measures are generally used to assess intestinal structure integrity. The present study showed that hens fed with the diets containing MOA or MEO complex developed higher villus height-to-crypt depth ratio in the small intestine, which was similar to the results of Chen et al. [4, 8], Wang et al. [11], and Yarmohammadi Barbarestani et al. [12]. The mechanism through which MOA and MEO complexes inclusion improved the intestinal morphology has been associating with their ability to suppress the intestinal pathogenic bacteria, which may improve the ability of intestinal epithelial cells to regenerate villus and reduce damage to villi caused by harmful bacteria and toxins [10].

Intestinal AKP and diglucosidase are major expressed in the brush border of intestinal mucosal epithelial cells, and their activities are important indicators for evaluating the intestinal epithelial cells maturation and intestinal functions. Increasing diglucosidase activity would benefit the digestion of carbohydrate in the intestinal tract, contributing to increase the feed efficiency. The present study showed that dietary both MOA and MEO complexes contributed to an improvement in the suctase activity in the ileal mucosa of laying hens. In addition, the MEO complex supplementation enhanced the AKP activity of the ileum. Similarly, some studies have revealed that dietary carvacrol EO, MMT, or EO and OA compounds enhanced the disaccharidase activity in the intestinal mucosa of laying hens or broiler chickens [10, 26, 27]. The MOA and MEO complexes increase the activity of brush border enzyme in the intestinal mucosa, and the underlying mechanism may be explained as follows: for the cation exchange capacity of MMT, the metal cations, including copper (Cu^{2+}), zinc (Zn^{2+}), ferrum (Fe^{3+}), and calcium

(Ca^{2+}), contained in the crystal layer of MMT may be replaced in the intestinal tract, then responsible for activating the brush border enzymes. Furthermore, the secretion of diglucosidase depends on the condition of intact intestinal mucosa morphology, for the reason why the MOA and MEO complexes may increase the activity of diglucosidase through reducing the numbers of intestinal harmful bacteria and improving the gut morphology.

The tight junctions in the gut are primarily composed by 3 kinds of transmembrane proteins: claudins, occludin, and junctional adhesion molecules. The claudin family and occludin are the main components in the regulation of intestinal epithelial physical barrier functions [28]. Redistribution or relocation of the tight junctions in the intestine will cause destruction of the intestinal barrier structure and increase in gut permeability [28, 29]. In this study, inclusion of MOA complex in diets increased the claudin-5 mRNA expression of the ileum. Similarly, our previous study has shown that dietary supplementation with MMT up-regulated the mRNA expression levels of occludin, claudin-1, and claudin-5 of the jejunum in laying hens [4, 8]. And the OA has also been demonstrated to regulate the genes expression of tight junction proteins in the intestine of poultry [10, 30]. To sum up, the improved gut morphology, enhanced the brush border enzyme activity, or increased the mRNA expression of claudin-5 in the ileum found in this study, indicating that dietary supplementation with both MOA and MEO complexes partially improved the gut physical barrier function, which may contribute to the health of intestine in laying hens and then offer benefits to the partially increase in egg production finally.

Conclusions

In conclusion, dietary supplementation with MOA or MEO complex decreased the amounts of harmful bacteria and the mRNA expression levels of inflammatory mediators, enhanced the brush border enzyme activity of the ileum, and partially improved the gut morphology in laying hens. In addition, dietary MOA complex up-regulated the claudin-5 mRNA expression in the ileum of laying hens. Above all, the results of this study indicated that both MOA and MEO complexes improved the intestinal health status in laying hens, which may contribute to the partially increase in the hens' laying performance.

Declarations

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Availability of data and material

The datasets supporting the conclusions of the present article are included within this article.

Authors' contributions

JC conceived and designed the experiments. JC and XG performed the experiments. JC analyzed the data. JC, XG and AA contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol for this study was approved by the Institutional Animal Care and Use Committee of Yichun University.

Competing interests

The authors confirm they have read Biomed Central's guidelines on competing interests and declare no competing interests.

Author details

College of Life Science and Resources and Environment, Yichun University, Yichun, Jiangxi, 336000, China

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