

# Effects of encapsulated essential oils and organic acids on laying performance, egg quality, intestinal morphology, barrier function, and microflora count of hens during the early laying period

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**ABSTRACT** The objective of this study was to investigate the effect of encapsulated essential oils and organic acids (EOA) on the growth performance, egg quality, intestinal morphology and functions, and microbial count of laying hens from week 21 to 30. A total of five hundred and four 21-wk-old layers were randomly allotted into 4 groups consisting of 7 replicates with 18 birds per replicate. The birds were fed a basic diet (CON) or diets with EOA at 150 mg/kg, 300 mg/kg, and 450 mg/kg in the other 3 groups, respectively. Compared to the CON group, the addition of 150 mg/kg EOA significantly increased laying rate ( $P < 0.05$ ) of hens from week 21 to 25. A linear increasing (linear,  $P < 0.01$ ) in ileal villus height of laying hens fed EOA from 150 to 300 mg/kg was observed at week 30. At week 25, the supplementation of 300 mg/kg EOA significantly increased ( $P < 0.05$ ) mRNA relative expression of aminopeptidase, sodium-glucose cotransporter 1, and

Na<sup>+</sup>-independent neutral amino acid transporter in duodenum and glucose transporter 2 in jejunum of laying hens compared to the CON groups. Meanwhile, the relative expression of glucose transporter 2 mRNA in the jejunum was upregulated with increasing concentration of EOA in diets (linear,  $P < 0.05$ ). Hens in EOA 300 group had higher mRNA relative expression of mucin-2 in ileum ( $P < 0.05$ ) than hens in CON group. Additionally, the secretory immunoglobulin in ileum A were linearly decreased (linear,  $P < 0.01$ ) with the increasing supplement of EOA. Dietary supplementation with EOA tended to increase ( $P = 0.083$ ) the counts of *Bifidobacterium* in cecal digesta at week 25 and 30. In conclusion, dietary with EOA may maintain intestinal tract morphology and promote digestive and absorptive capacities and barrier function, especially at 300 mg/kg. This study provided evidence of using EOA as a potential feed additive for laying hens.

**Key words:** essential oil, gut microflora, intestinal function, laying hen, organic acid

2019 Poultry Science 98:6751–6760  
<http://dx.doi.org/10.3382/ps/pez391>

## INTRODUCTION

Antibiotics have been used as growth promoter for a long time, but the microbial resistance and drug residue issues are more sharply at current. What's more, the antibiotic growth promoter was banned to be used as feed additive of layers during egg producing period (Donoghue, 2003; Castanon, 2007). With the demands for high quality poultry products, it is imperative to exploit the effective and green feed additives that can stimulate the latent productive capacity of laying hens. When hens are in the laying period, the reproductive system develops fast and nutrient requirement increases markedly to meet the growth and production needs, especially for the 3 essential nutrients (carbohydrate,

fat, and protein). Meanwhile, limestone powder is used in feed for the calcium requirement of laying hens, but the pH value of gastrointestinal tract will be influenced with the acids-binding capacity of limestone powder. Consequently, as the pH value increased, the activity of digestive enzymes reduced and reproducing of the intestinal pathogens accelerated, to the extent that the efficient utilization of nutrients is decreased and the risk of suffering from gut disease increased (Walk et al., 2012; Paiva et al., 2014).

Organic acids are carbon-containing acid compounds (Broom, 2015) and are known to be capable of decreasing the pH of intestinal digesta, improving the growth performance (Aclkgoz et al., 2011; Khan and Iqbal, 2016), maintaining the intestinal morphology, keeping the balance of intestinal flora, increasing the digestive enzymes activity and utilization of mineral (Senkoylu et al., 2007; Andreopoulou et al., 2014; Wu et al., 2016), and also stimulating the immune function (Emami et al., 2013; Lee et al., 2017). Essential oils are aromatic compounds extracted from plants, and are widely

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Received March 16, 2019.

Accepted June 13, 2019.

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used as food additives because of the bacteriostasis and fragrance characters. As a new type of feed additives, thymol (an essential ingredient of essential oils) has a better antibacterial effect on animal production. Furthermore, thymol can keep intestinal microflora stabilization and has an effective anti-inflammatory and antioxidant property (Yu et al., 2018). Finally, essential oils, including thymol, can keep the gut and host healthy and improve the performance of animals (Jang et al., 2007; Brenes and Roura, 2010). They all really point to us that organic acids and essential oils have positive functions on intestinal health. The small intestine is the main place for digestion and absorption of nutrients, and digestive enzymes and nutrition transporters play a significant role in the procession of digestion and absorption (Mott et al., 2008). There is a large amount of gastric acid and bile salts in the foregut of the chicken, and the microbial balance is not easily destroyed. However, insufficiency of gastric acid and bile salts secretion may alter the microbial homeostasis of the hindgut. Therefore, encapsulated essential oils and organic acids (EOA) could be used as a local slow release additive in the distal intestinal tract of laying hens.

Based on our previous experiments of EOA on broilers, which reduced harmful bacteria, promote digestive enzyme activity, and increased the feed conversion ratio of broilers (Yang et al., 2019), this experiment was to evaluate a combination diet of EOA on epithelial restitution, intestinal digestion and absorption, and microflora count of hens during early laying period.

## MATERIALS AND METHODS

### Birds and Experimental Design

All the birds and experimental protocol in this study were approved by the Institution Animal Care and Use Committee of the Northwest A&F University (Protocol number: NWFAC1008).

Briefly, a total of 504 healthy Roman laying hens with similar weights at 21 wk of age were randomly assigned to 4 experimental groups. Each group had 7 replicates and each replicate contained 18 birds. The body weight of the hens was  $1.63 \pm 0.039$  kg and there was no significant difference between groups. A total of 168 stainless steel 4-layer semi-stacked cages (38 cm-width  $\times$  52 cm-length  $\times$  40 cm-height), with 3 hens assigned randomly to each, were used. The indoor environment was kept at  $22 \pm 2^\circ\text{C}$ , the daily lighting time was 16 hr and 8 hr in darkness. The birds were fed a basic diet (CON) or diets with EOA at 150 mg/kg, 300 mg/kg and 450 mg/kg (EOA 150, 300, and 450) in the other 3 groups, respectively. The composition of the experimental diets is shown in Table 1. The feeding experiment lasted for 10 wk, the birds had free access to feed and water. The compounds EOA (containing a minimum of 200 g/kg of sorbic acid, a minimum of 200 g/kg fumaric acid, and a minimum of 100 g/kg thymol) was provided by Jefe Nutrition Inc., St-Hyacinthe, Quebec, Canada.

**Table 1.** Composition and nutrient levels of basal diet (as-fed basis).<sup>1</sup>

Item (%)	Content
Ingredient	
Corn	66.00
Soybean meal	17.20
Cottonseed meal	4.00
Corn gluten powder (CP 60%)	2.00
Limestone	5.20
Large granular calcium	4.00
CaHPO <sub>4</sub>	0.60
NaCl	0.20
Baking soda	0.20
Lysine sulfate	0.20
DL-methionine	0.16
Choline chloride	0.10
Phytase (5000IU)	0.01
Multi-vitamin <sup>2</sup>	0.03
Iodine selenium mixture	0.10
Calculated nutrition	
Metabolizable energy (MJ/kg)	11.13
Available phosphorus	0.35
Analyzed nutrition	
Crude protein	15.74
Calcium	3.85
Total phosphorus	0.48
Lysine	0.84
Methionine	0.41
Methionine + cysteine	0.66
Threonine	0.61

<sup>1</sup>The 3 experimental diets were the basal diet supplemented with the encapsulated essential oils and organic acids at 150 mg/kg, 300 mg/kg, and 450 mg/kg, respectively.

<sup>2</sup>The vitamin premix provided per kg of diets: vitamin A, 250,000 IU; vitamin D, 50,000 IU; vitamin K3, 53 mg; vitamin B1, 40 mg; vitamin B2, 120 mg; vitamin B12, 0.50 mg; vitamin E, 600 IU; biotin, 0.65 mg; folic acid, 25 mg; pantothenic acid, 240 mg; and niacin, 1,000 mg.

### Performance and Samples Collection

Feed disappearance and mortalities were recorded and the hens were weighed per replicate by period and for the entire experiment. During the experiment, the egg numbers from each replicate and were recorded and eggs were weighted daily. Then average daily feed intake (ADFI, feed intake per day per hen), average daily egg weight (ADEW, egg weight per day per hen), feed-egg ratio (F/E, ADFI/ADEW), and laying rate (the number of eggs/the number of hens) were calculated by period and cumulatively. A total of 14 fresh eggs were randomly selected from each replicate from eggs produced the last day at week 25 and 30 of age for egg quality testing, including the eggshell thickness (EST, ETG-1601A, Robotmation, Japan), eggshell strength (ESS, EFG-0503, Robotmation, Japan), albumen height (AH), yolk color (YC), and Haugh unit (HU, EMT-5200, Japan).

At 25 and 30 wk of age, 1 bird in each replicate, with the nearly average body weight, was killed, and the duodenum, jejunum, ileum, and cecum were collected. After the intestinal contents were removed with pre-cooling saline, intestinal segments long for 1 cm from the middle of the duodenum, jejunum, and ileum were collected and fixed in 4% paraformaldehyde solution and stored in a refrigerator at  $-4^\circ\text{C}$  for intestinal morphology measurements. Duodenum and jejunum were longitudinally

sectioned and intestinal mucosa was scraped with a sterile glass slide for detection of mRNA expression of digestive enzyme, nutrient transporters, and intestinal tract barrier factors, also sIgA secretion. Contents of the cecum were collected into the sterile microtube for microbiota population count. Then samples of intestinal mucosa and contents of the cecum were placed in liquid nitrogen and transferred to a  $-80^{\circ}\text{C}$  freezer subsequently.

### Intestinal Morphology

After dehydrated, transparented, sliced, haematoxylin eosin stained, and sheet sealed, each slice was chosen with 10 visions for detecting the intestine villus height (**VH**), crypt depth (**CD**) and intestinal wall thickness (**WT**) using phase contrast microscope, and villus height/crypt depth (**V/C**) was calculated. The detailed procedures were referenced from Naghi Shokri et al. (2017).

### RNA Isolation, cDNA Synthesis, and Real Time PCR

The method of RNA extraction and the procession of reverse transcription and real time PCR referred

to reported ways (Liu et al., 2016). Total RNA of the duodenum and jejunum mucosa was isolated using Trizol Reagent according to the manufacture's protocols (TaKaRa, Dalian, China). The concentration of RNA was determined at the absorbance of 260 nm by Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, Delaware), and the absorbance for the extraction was between 1.8 and 2.0 at A260/A280. The mRNA was reversed transcribed into cDNA by Primer Script RT reagent Kit (TaKaRa, Dalian, China) according to the procedures of the manufacturers. Gene expression of mucosa enzyme and nutrition transporters was analyzed by real-time PCR (Bio-Rad, California, USA). Gene expression was finally normalized to  $\beta$ -actin and the relative expression of each gene was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method. The primers for analysis of gene expression of maltase, sucrase (Speier et al., 2012), aminopeptidase, sodium-glucose cotransporter 1 (**SGLT1**, or **SLC5A1**), glucose transporter 2 (**GLUT2**, or **SLC2A2**) (Liu et al., 2015),  $y^+$ L amino acid transporter-2 ( $y^+$ **LAT2**, or **SLC7A6**),  $\text{Na}^+$ -independent neutral amino acid transporter ( $b^{0+}$ **AT**, or **SLC7A9**),  $\text{Na}^+$ ,  $\text{Cl}^-$ -dependent neutral and cationic amino acid transporter (**ATB** $^{0+}$ , or **SLC6A1**), and fatty acid transporter1 (**FAPT1**, or **SLC27A1**) (Yuan et al., 2012) are shown in Table 2.

**Table 2.** Sequences of primers for quantitative real-time PCR assay.<sup>1</sup>

Gene name/abbreviation	Accession number	Primer sequences (5' to 3')
<i>GAPDH</i>	L08165	F:AGAACATCATCCCAGCGTCC R:CGGCAGGTCAGGTCAACAAC
Aminopeptidase	NM_204,861	F:TTGGCAACAAGGAGCGAGTG R:AGTGGGTGGGAGGTGGTCAG
Maltase	XM_01,527,3018	F:ACGCTCACGGGCAGAAATAC R:GCACTCCTCTATCCACCAACG
Sucrase	XM_01,529,1762	F:CGCAAAAGCACAGGGACAGT R:TCGATACGTGGTGTGTGCTCAGTT
<i>GLUT2</i>	NM_205,209	F:CACACTATGGGCGCATGCT R:ATTGTCCCTGGAGGTGTTGGTG
<i>SGLT1</i>	NM_0,012,93240	F:AGCATTTCAGCATGGTGTGTCTTC R:GATGCTCCTATCTCAGGGCAGTTC
<i>ATB</i> $^{0+}$	XM_414,303	F:TTAACCCCTCTTGTGCCGTT R:ATTGAAGTCCTTCTTTCTCGCC
$b^{0+}$ <i>AT</i>	NM_0,011,99133	F:AGGTGGGCTGATTAGTGGA R:AGTGCACCTAGTGTGGCCAG
$y^+$ <i>LAT2</i>	XM_413,988	F:CCTGATAGTAGGCAACAT R:AGAACAAGGCAGAGTAGAG
<i>FAPT1</i>	NC_0,06088	F:GACTGCGCCAAGTACAGATGC R:CACTCGGTGGCTCCGTAGAAC
Mucin-2	XM_421,035	F:TTTCATGATGCCTGCTCTTGTG R:CCTGAGCCTTGGTACATTCTTGT
Occludin-1	NM_205,128	F:ACGGCAGCACCTACCTCAA R:GGGCGAAGAAGCAGATGAG
<i>ZO-1</i>	XM_015,2789	F:TATAGAAGATCGTGCGCCTCC R:GAGGTCTGCCATCGTAGCTC

<sup>1</sup> *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse; *GLUT2*, Glucose transporter 2; *SGLT1*, Sodium -dependent glucose cotransporter 1; *ATB* $^{0+}$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$ -dependent neutral and cationic amino acid transporter;  $b^{0+}$ *AT*,  $\text{Na}^+$ -independent neutral amino acid transporter;  $y^+$ *LAT2*,  $y^+$ L amino acid transporter 2; *FAPT1*, Fatty acid transporter 1; and *ZO-1*, zona occludens 1.

## Secretory Immunoglobulin A

After intestinal mucosa was defrosted, homogenized and centrifuged, the secretory immunoglobulin A (sIgA) levels were determined by ELISA (China Institute of Atomic Energy, Beijing, China). Protein contents of intestinal mucosa were measured by the Bradford method.

## DNA Isolation and Real Time PCR

Before the isolation of DNA, the cecum contents samples were fully mixed for every microtube under low-temperature environment. Genomic DNA was isolated from 50 mg chyme using Easy Pure Genomic DNA Kit according to the manufacture's protocols (Trans Gen, Beijing, China). The quality of the DNA was measured with agarose gel (1%) electrophoresis and the concentration was detected using Nanodrop 2000c spectrophotometer at 260 and 280 nm (Thermo Fisher Scientific Inc., Wilmington, Delaware). And the DNA was stored at  $-80^{\circ}\text{C}$  for future analysis.

The standard curve preparation was based on the previous method (Taverniers et al., 2005; Liu et al., 2017). The population of cecal microbiota was determined by SYBR green-based absolute quantitative real-time PCR (Bio-Rad, California, USA) and the primer used for detecting *Lactobacillus*, *Bifidobacterium*, *Escherichia Coli* and *Salmonella* (Schwiertz et al., 2010) are listed in the Table 3. The amplification system for qPCR was 20  $\mu\text{L}$  mixture consisted of 10  $\mu\text{L}$  of SYBR Premix Ex Taq (Takara, Dalian, China), 1  $\mu\text{L}$  of each primer (10  $\mu\text{mol/L}$ ), 1  $\mu\text{L}$  of the extracted bacterial genomic DNA (20 ng/ $\mu\text{l}$ ), and 7  $\mu\text{L}$   $\text{H}_2\text{O}$ . The conditions of qPCR reaction were:  $95^{\circ}\text{C}$  for 30 s, followed by 40 cycles of  $95^{\circ}\text{C}$  for 5 s,  $60^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 30 s. The relative proportion values were calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method. Finally, according to the standard curve, the number of copies of bacteria in the sample was calculated. The calculation formula was:  $X = \lg[(\text{Mdna}/20 \times \text{C20}/\text{Mc})]$  (Mdna: the total weight of the sample DNA; Mc: weight of sample contents; C20: copy number of every 20 ng of DNA sample). Results were reported as equivalent log 10 cfu per DNA concentration.

**Table 3.** Sequences of the primers used for the determination of the microbial populations.

Name	Primer sequences (5' to 3')
<i>Lactobacillus</i>	F:CACCGCTACACATGGAG R:AGCAGTAGGGAATCTTCCA
<i>Salmonella</i>	F:GGAGTCTTGTAGAGGGGGGT R:AGGTAAGGTTCTTCGCGTTG
<i>Bifidobacterium</i>	F:CCACCGTTACACCGGGAA RGGGTGGTAATGCCGGATG
<i>Escherichia coli</i>	F:CGGGTAACGTCAATGAGCAAA R:CATGCCGCGTGTATGAAGAA

## Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 20.0 statistic software (SPSS Institute Inc., Chicago, Illinois). Differences between treatments were determined by Tukey's multiple comparisons test. Orthogonal polynomial contrast coefficients were used to determine the linear and quadratic effect of increasing level of dietary EOA on the measured traits. The criterion of statistically significant for all data was at  $P$ -value less than 0.05 ( $P < 0.05$ ).

## RESULTS AND DISCUSSION

### Performance

Numerous studies have revealed extensive functions of organic acids and essential oil when applied in animal production (Broom, 2015; Jiang et al., 2015; Zeng et al., 2015). The body weight of the hens was  $1.93 \pm 0.053$  kg at the end of the experiment and no significant difference were found in body weight and mortality rate among each treatment (data not shown). Adding EOA to the diet had no significant influence on ADEW and F/E ( $P > 0.05$ ) compared to the CON group (Table 4). Laying rate was significantly increased ( $P < 0.05$ ) by 150 mg/kg EOA from week 21 to 25. Birds in EOA 150 group showed a lower ADFI (quadratic,  $P < 0.01$ ; ANOVA,  $P < 0.05$ ) than birds in EOA 300 and 450 groups from week 21 to 30. Previous studies (Gheisar et al., 2014; Habibi et al., 2014; Du et al., 2016) had shown conflicting results in the effects of organic acids and essential oils on growth performance may be influenced by their type and dose, different dietary compositions, the environment, management, and age differences (Zeng et al., 2015).

### Egg Quality

The EOA 300 and 450 groups showed higher ESS ( $P < 0.05$ ), and the EOA 150 and 450 groups showed higher YC ( $P < 0.05$ ) compared to the CON group at week 30 (Table 5). Yolk color almost depends on the consumption of pigmented substances (such as carotenoids) from the feed (Lessire et al., 2017). The main components of EOA are thymol, sorbic acid, and fumaric acid which may keep the fat-soluble components. Thus, the carotenoids and vitamin D accumulated in egg should contribute to the increasing of YC in the EOA groups. Kang et al. (2016) showed that ESS was significantly increased after the addition of vitamin  $\text{D}_3$ . As no extra vitamin  $\text{D}_3$  and calcium were added to the diet of EOA groups, the significantly increased ESS may be the results of the enhanced deposition of calcium based on the addition of EOA.

**Table 4.** Effects of encapsulated essential oils and organic acids (EOA) on the growth performance of laying hens.<sup>1</sup>

Item	CON	EOA (mg/kg)			SEM	ANOVA	P-value	
		150	300	450			Linear	Quadratic
Week 21 to 25								
ADFI (g)	111.90 <sup>a,b</sup>	108.13 <sup>b</sup>	112.89 <sup>a</sup>	113.16 <sup>a</sup>	0.628	0.007	0.072	0.057
ADEW (g)	54.14	54.32	54.34	54.02	0.115	0.737	0.577	0.870
F/E (g/g)	2.20	2.17	2.16	2.19	0.008	0.104	0.062	0.690
Laying rate (%)	92.59 <sup>b</sup>	95.05 <sup>a</sup>	94.39 <sup>a,b</sup>	94.71 <sup>a,b</sup>	0.349	0.047	0.052	0.099
Week 26 to 30								
ADFI (g)	117.56	115.23	118.14	116.70	0.454	0.128	0.063	0.022
ADEW (g)	56.96	57.04	56.71	56.63	0.162	0.806	0.909	0.687
F/E (g/g)	2.39	2.33	2.42	2.35	0.014	0.115	0.059	0.020
Laying rate (%)	89.29	88.77	89.45	87.60	0.416	0.413	0.255	0.436
Week 21 to 30								
ADFI (g)	113.16 <sup>a,b</sup>	110.82 <sup>b</sup>	114.91 <sup>a</sup>	114.54 <sup>a</sup>	0.522	0.018	0.028	0.012
ADEW (g)	55.40	55.76	55.47	55.22	0.129	0.574	0.578	0.573
F/E (g/g)	2.30	2.27	2.30	2.29	0.009	0.598	0.411	0.231
Laying rate (%)	91.57	91.38	92.21	91.73	0.284	0.792	0.671	0.404

<sup>a,b</sup>Mean values with the row with different superscript letter was significant difference ( $P < 0.05$ ).

<sup>1</sup>CON, basal diet; EOA, basal diet with encapsulated organic acids and essential oils; SEM, standard error of mean; ADFI, average daily feed intake; ADEW, average daily egg weight; and F/E, feed-egg ratio.

**Table 5.** Effects of encapsulated essential oils and organic acids (EOA) on the egg quality of laying hens.<sup>1</sup>

Item	CON	EOA (mg/kg)			SEM	ANOVA	P-value	
		150	300	450			Linear	Quadratic
Week 25								
EST (mm)	0.41	0.38	0.38	0.39	0.005	0.153	0.221	0.099
ESS (kg/cm <sup>2</sup> )	5.57	5.19	5.59	5.76	0.110	0.320	0.331	0.221
AH (mm)	7.38	7.01	7.37	7.51	0.145	0.382	0.390	0.581
YC	12.82	12.25	11.97	12.54	0.137	0.143	0.095	0.632
HU	84.97	88.75	87.01	89.47	0.706	0.112	0.248	0.109
Week 30								
EST (mm)	0.37	0.39	0.40	0.39	0.005	0.192	0.315	0.648
ESS (kg/cm <sup>2</sup> )	5.17 <sup>c</sup>	5.56 <sup>b,c</sup>	5.86 <sup>a,b</sup>	6.28 <sup>a</sup>	0.131	0.018	0.974	0.827
AH (mm)	6.06	5.39	5.91	5.89	0.169	0.544	0.993	0.358
YC	12.31 <sup>b</sup>	13.18 <sup>a</sup>	12.75 <sup>a,b</sup>	13.13 <sup>a</sup>	0.107	0.005	0.022	0.014
HU	76.49	71.74	76.14	76.71	1.194	0.419	0.639	0.277

<sup>a-c</sup>Mean values with the row with different superscript letter was significant difference ( $P < 0.05$ ).

<sup>1</sup>n = 7 per treatment group; CON, basal diet; EOA, basal diet with encapsulated organic acids and essential oils; SEM, standard errors of mean; EST, eggshell thickness; ESS, eggshell strength; AH, albumen height; YC, yolk color; and HU, Haugh unit.

## Intestinal Morphology

With an increase in concentration of EOA in diets, WT in jejunum (quadratic,  $P < 0.001$ ; ANOVA,  $P < 0.01$ ), and VH in ileum (linear,  $P < 0.01$ ; ANOVA,  $P < 0.01$ ) of laying hens were increased at week 30 (Table 6). Previous study manifested that organic acid mixture (at least 60% formic acid) had a positive effect on the intestinal morphology (Kaya et al., 2015). The result showed that EOA had a significant effect on the morphology of the hindgut intestine chiefly because EOA is a coated product to realize protection of biological activity and sustained release.

## Expression of Digestive Enzymes and Nutrient Transporters

Essential oils and organic acid supplementation could increase the nutrient digestibility (Iqbal et al., 2019).

In our current study, mRNA relative expression of the aminopeptidase (linear and quadratic,  $P < 0.01$ ; ANOVA,  $P < 0.01$ ), and maltase (quadratic,  $P < 0.01$ ; ANOVA,  $P < 0.05$ ) in duodenum mucosa upregulated with increasing concentration of EOA in diets at week 25 (Table 7). The relative expression of *GLUT2* mRNA in the jejunum upregulated with increasing concentration of EOA in diets (quadratic at week 25,  $P < 0.001$ ; linear at week 30,  $P < 0.05$ ), and it was significantly higher ( $P < 0.05$ ) in birds fed diet contained 150 and 300 mg/kg EOA at week 25 and 450 mg/kg EOA at week 30 compared to control birds (Table 8). Dietary supplementation with 300 mg/kg EOA boosted ( $P < 0.05$ ) the relative expression of *b<sup>0,+</sup>AT* and *SGLT1* in duodenum at week 25. The higher mRNA expression of digestion enzyme and transporter protein reflects the improving of intestinal morphology and function. The increasing maltase expression could facilitate the digestive of carbohydrate and accompany by increasing

**Table 6.** Effects of encapsulated essential oils and organic acids (EOA) on the intestinal morphology of laying hens.<sup>1</sup>

Item	CON	EOA (mg/kg)			SEM	P-value			
		150	300	450		ANOVA	Linear	Quadratic	
Week 25									
Duodenum	VH ( $\mu\text{m}$ )	994.82	1,077.74	1,032.23	1,051.33	31.322	0.884	0.666	0.638
	CD ( $\mu\text{m}$ )	61.38	53.71	55.04	61.00	1.526	0.173	0.987	0.031
	V/C	16.35	22.23	18.74	17.30	0.625	0.149	0.789	0.037
	WT ( $\mu\text{m}$ )	125.88	124.79	145.79	120.77	4.730	0.280	0.887	0.215
Jejunum	VH ( $\mu\text{m}$ )	735.06	747.15	704.46	683.42	26.564	0.839	0.428	0.768
	CD ( $\mu\text{m}$ )	64.69	56.88	60.54	55.04	1.697	0.179	0.091	0.726
	V/C	11.41	13.12	11.62	12.45	0.359	0.313	0.609	0.544
	WT ( $\mu\text{m}$ )	124.16	100.64	119.67	127.19	5.425	0.289	0.562	0.160
Ileum	VH ( $\mu\text{m}$ )	631.28	657.24	621.65	611.79	31.800	0.966	0.761	0.793
	CD ( $\mu\text{m}$ )	49.41	57.47	52.59	57.69	2.172	0.505	0.328	0.740
	V/C	12.64	11.38	11.85	10.54	0.306	0.107	0.035	0.963
	WT ( $\mu\text{m}$ )	139.28	128.51	149.03	151.06	7.697	0.729	0.449	0.692
Week 30									
Duodenum	VH ( $\mu\text{m}$ )	1,028.84	1,059.74	1,054.13	1,059.01	28.014	0.981	0.963	0.865
	CD ( $\mu\text{m}$ )	46.67	52.61	52.97	53.71	1.167	0.135	0.041	0.244
	V/C	20.98	19.41	19.90	19.77	0.624	0.855	0.783	0.658
	WT ( $\mu\text{m}$ )	114.28	133.97	122.20	128.20	3.874	0.354	0.385	0.386
Jejunum	VH ( $\mu\text{m}$ )	849.83	902.71	855.07	903.11	24.589	0.820	0.642	0.964
	CD ( $\mu\text{m}$ )	55.38	54.24	50.24	48.54	1.324	0.217	0.896	0.654
	V/C	16.44	15.94	16.97	18.67	0.586	0.405	0.642	0.872
	WT ( $\mu\text{m}$ )	108.40 <sup>b,c</sup>	134.78 <sup>a</sup>	96.69 <sup>c</sup>	118.96 <sup>a,b</sup>	4.333	0.002	0.001	<0.001
Ileum	VH ( $\mu\text{m}$ )	568.37 <sup>b</sup>	587.62 <sup>b</sup>	752.36 <sup>a</sup>	595.24 <sup>b</sup>	23.00	0.002	0.002	0.010
	CD ( $\mu\text{m}$ )	39.73	44.13	44.73	43.81	1.559	0.679	0.404	0.429
	V/C	17.69	14.73	16.64	14.68	0.796	0.441	0.204	0.336
	WT ( $\mu\text{m}$ )	115.93	136.51	122.33	128.02	4.093	0.350	0.568	0.374

<sup>a-c</sup>Mean values with the row with different superscript letter was significant difference ( $P < 0.05$ ).

<sup>1</sup> $n = 7$  per treatment group; CON, basal diet; EOA, basal diet with encapsulated organic acids and essential oils; SEM, standard errors of mean; VH, villus height; CD, crypt depth; V/C, villus height: crypt depth; and WT, intestinal wall thickness.

**Table 7.** Effects of encapsulated essential oils and organic acids (EOA) on sucrase, aminopeptidase, and maltase mRNA relative expression in intestinal mucosa of laying hens.<sup>1</sup>

Item	CON	EOA (mg/kg)			SEM	P-value			
		150	300	450		ANOVA	Linear	Quadratic	
Week 25									
Duodenum	Aminopeptidase	0.13 <sup>b</sup>	0.14 <sup>b</sup>	0.71 <sup>a</sup>	0.23 <sup>b</sup>	0.076	0.003	0.003	0.003
	Maltase	1.21 <sup>a,b</sup>	0.07 <sup>b</sup>	0.10 <sup>b</sup>	2.38 <sup>a</sup>	0.314	0.010	0.100	0.003
	Sucrase	0.82	0.93	1.44	1.09	0.113	0.266	0.177	0.315
Jejunum	Aminopeptidase	1.11	0.83	0.90	0.62	0.140	0.178	0.045	0.379
	Maltase	1.11	1.23	0.72	1.08	0.163	0.355	0.840	0.872
	Sucrase	1.24 <sup>a,b</sup>	0.71 <sup>b</sup>	2.30 <sup>a</sup>	1.41 <sup>a,b</sup>	0.195	0.041	0.145	0.593
Week 30									
Duodenum	Aminopeptidase	1.81	0.82	1.29	1.26	0.198	0.417	0.501	0.247
	Maltase	1.53	1.55	1.27	1.04	0.147	0.661	0.804	0.272
	Sucrase	0.42	1.01	1.66	1.18	0.258	0.459	0.259	0.332
Jejunum	Aminopeptidase	0.90	1.46	0.82	1.06	0.122	0.270	0.150	0.068
	Maltase	0.80	1.36	0.77	1.07	0.118	0.252	0.140	0.060
	Sucrase	1.88	1.72	1.50	2.12	0.230	0.500	0.144	0.576

<sup>a,b</sup>Mean values with the row with different superscript letter was significant difference ( $P < 0.05$ ).

<sup>1</sup> $n = 7$  per treatment group; CON, basal diet; EOA, basal diet with encapsulated organic acids and essential oils; and SEM, standard error of mean.

of glucose concentration. Accordingly, upregulation of *GLUT2* that is located in the basolateral membrane contributed to transfer glucose into bloods. Therefore, the trend of improved F/E in the early stage of the trial may be partly due to the increased energy utilization of the mucosa after EOA supplemented, and the improved yolk color of EOA addition also found the reason of the

higher assimilation ability. In addition, aminopeptidase is responsible for cleaving amino acids from the N terminus of peptides (Miska et al., 2014), thus, the increasing of *b<sup>0,+</sup>AT* mRNA expression could uptake more dissociated amino acids, especially for methionine and lysine (Su et al., 2015). Therefore, the digestion and absorption efficiency of carbohydrate and protein improved at

**Table 8.** Effects of encapsulated essential oils and organic acids (EOA) on mRNA relative expression of intestinal nutrient transporters in intestinal mucosa of laying hens.<sup>1</sup>

Item	CON	EOA (mg/kg)			SEM	P-value			
		150	300	450		ANOVA	Linear	Quadratic	
Week 25									
Duodenum	<i>GLUT2</i>	0.74	1.12	0.90	1.19	0.104	0.452	0.260	0.825
	<i>SGLT1</i>	0.71 <sup>b</sup>	1.18 <sup>a,b</sup>	1.53 <sup>a</sup>	0.82 <sup>a,b</sup>	0.112	0.026	0.404	0.005
	<i>ATB<sup>0,+</sup></i>	0.94	0.81	0.47	1.53	0.149	0.132	0.266	0.048
	<i>b<sup>0,+</sup>AT</i>	0.61 <sup>b</sup>	1.13 <sup>a,b</sup>	1.62 <sup>a</sup>	0.99 <sup>a,b</sup>	0.125	0.015	0.063	0.937
	<i>y<sup>+</sup>LAT2</i>	1.01	0.83	0.68	1.29	0.110	0.236	0.158	0.458
	<i>FAPT1</i>	1.01	0.87	0.66	1.15	0.087	0.316	0.791	0.094
Jejunum	<i>GLUT2</i>	0.53 <sup>b</sup>	1.30 <sup>a</sup>	1.21 <sup>a</sup>	0.52 <sup>b</sup>	0.111	0.001	0.862	<0.001
	<i>SGLT1</i>	1.52 <sup>a</sup>	0.42 <sup>b</sup>	1.76 <sup>a</sup>	0.83 <sup>b</sup>	0.161	0.003	0.433	0.694
	<i>ATB<sup>0,+</sup></i>	1.83	2.36	1.17	1.79	0.236	0.455	0.537	0.923
	<i>b<sup>0,+</sup>AT</i>	3.68	2.60	2.27	3.68	0.267	0.119	0.873	0.024
	<i>y<sup>+</sup>LAT2</i>	0.84	1.67	0.96	0.97	0.191	0.431	0.846	0.317
	<i>FAPT1</i>	1.45	0.94	1.17	1.11	0.074	0.065	0.169	0.100
Week 30									
Duodenum	<i>GLUT2</i>	0.94	0.89	1.21	1.54	0.138	0.339	0.740	0.775
	<i>SGLT1</i>	1.62	1.94	1.90	1.77	0.163	0.920	0.800	0.533
	<i>ATB<sup>0,+</sup></i>	1.39	0.95	1.06	0.76	0.094	0.098	0.405	0.209
	<i>b<sup>0,+</sup>AT</i>	1.00	0.79	1.25	0.88	0.092	0.359	0.899	0.659
	<i>y<sup>+</sup>LAT2</i>	1.42	1.38	0.87	1.05	0.113	0.262	0.455	0.254
	<i>FAPT1</i>	1.45	0.94	1.17	1.11	0.074	0.065	0.169	0.100
Jejunum	<i>GLUT2</i>	0.63 <sup>b</sup>	0.72 <sup>b</sup>	0.78 <sup>b</sup>	1.23 <sup>a</sup>	0.069	0.001	0.037	0.658
	<i>SGLT1</i>	0.48	1.02	1.05	0.92	0.089	0.099	0.080	0.054
	<i>ATB<sup>0,+</sup></i>	1.10	0.81	0.85	0.79	0.110	0.785	0.406	0.636
	<i>b<sup>0,+</sup>AT</i>	1.38	1.14	1.05	1.26	0.150	0.896	0.762	0.506
	<i>y<sup>+</sup>LAT2</i>	0.71	0.74	1.09	1.25	0.098	0.124	0.028	0.713
	<i>FAPT1</i>	1.45	0.94	1.17	1.11	0.074	0.065	0.169	0.100

<sup>a,b</sup>Mean values with the row with different superscript letter was significant difference ( $P < 0.05$ ).

<sup>1</sup>n = 7 per treatment group; CON, basal diet; EOA, basal diet with encapsulated organic acids and essential oils; SEM, standard errors of mean; *GLUT2*, Glucose transporter 2; *SGLT1*, Sodium -dependent glucose cotransporter 1; *ATB<sup>0,+</sup>*, Na<sup>+</sup>, Cl-dependent neutral and cationic amino acid transporter; *b<sup>0,+</sup>AT*, Na<sup>+</sup>-independent neutral amino acid transporter; *y<sup>+</sup>LAT2*, y<sup>+</sup>L amino acid transporter 2; and *FAPT1*, Fatty acid transporter 1.

**Table 9.** Effects of encapsulated essential oils and organic acids (EOA) on microbial count (log<sub>10</sub>) in cecal digesta of laying hens.<sup>1</sup>

Item	CON	EOA (mg/kg)			SEM	P-value		
		150	300	450		ANOVA	Linear	Quadratic
Week 25								
<i>Lactobacillus</i>	11.97	11.90	12.11	12.03	0.090	0.911	0.895	0.676
<i>Bifidobacterium</i>	8.86	9.51	9.22	8.85	0.110	0.083	0.978	0.309
<i>Escherichia Coli</i>	9.87	10.55	10.71	9.76	0.212	0.306	0.932	0.070
<i>Salmonella</i>	8.32	9.23	8.92	8.20	0.192	0.169	0.115	0.794
Week 30								
<i>Lactobacillus</i>	11.64	11.84	11.45	11.68	0.071	0.358	0.471	0.752
<i>Bifidobacterium</i>	9.31	9.86	10.47	9.60	0.167	0.083	0.333	0.047
<i>E. Coli</i>	9.20	8.86	10.11	9.27	0.196	0.177	0.371	0.516
<i>Salmonella</i>	8.93	8.37	9.11	8.53	0.186	0.502	0.806	0.977

<sup>1</sup>n = 7 per treatment group; SEM, standard error of mean; CON, basal diet; and EOA, basal diet with encapsulated organic acids and essential oils.

the same time. And the rise of amino acids transporter quantity contributed to better reproductive system development and performance of laying hens.

### Intestinal Microbial Status and Gut Barrier Function

Dietary supplementation with EOA tended to increase ( $P = 0.083$ ) the counts of *Bifidobacterium* in cecal digesta at week 25 and 30 (Table 9). The result was

similar to earlier findings, where adding with EOA had a positive impact on the gut microflora of the ileum (Liu et al., 2017; Adaszynska-Skwirzynska and Szczerbinska, 2019). The mixture of essential oils comprised of 25% thymol and 25% carvacrol increased ileum *Lactobacillus* population and reduces effect of necrotic enteritis caused by *Clostridium perfringens* in chickens (Yin et al., 2017). *Bifidobacteria* is the dominant microflora of the normal intestinal microflora, which can inhibit the growth of pathogenic bacteria and enhance the immune function of the body (Bottacini et al., 2017).

**Table 10.** Effects of encapsulated essential oils and organic acids (EOA) on mRNA relative expression of mucin-2, occludin, and zona occludens-1 in intestinal mucosa of laying hens.<sup>1</sup>

Item	CON	EOA (mg/kg)			SEM	P-value			
		150	300	450		ANOVA	Linear	Quadratic	
Week 25									
Duodenum	Mucin-2	0.85	0.67	0.88	1.08	0.114	0.731	0.414	0.447
	Occludin	1.31	1.01	0.93	0.87	0.131	0.684	0.277	0.698
	ZO-1	1.65	0.99	0.74	1.60	0.203	0.299	0.826	0.077
Jejunum	Mucin-2	0.82	0.63	0.50	0.66	0.087	0.689	0.516	0.361
	Occludin	0.79	1.17	0.76	1.13	0.095	0.288	0.479	0.960
	ZO-1	1.23	1.22	1.07	0.91	0.162	0.895	0.465	0.842
Ileum	Mucin-2	0.24 <sup>b</sup>	0.81 <sup>a,b</sup>	1.01 <sup>a</sup>	0.72 <sup>a,b</sup>	0.104	0.038	0.140	0.010
	Occludin	1.48	0.58	0.59	0.94	0.162	0.588	0.943	0.202
	ZO-1	0.56	0.86	1.75	0.66	0.187	0.100	0.445	0.056
Week 30									
Duodenum	Mucin-2	1.11	0.78	1.24	1.25	0.129	0.552	0.507	0.544
	Occludin	1.11	1.21	1.07	0.78	0.117	0.658	0.336	0.444
	ZO-1	0.86	1.00	0.69	0.81	0.070	0.486	0.493	0.947
Jejunum	Mucin-2	1.26	1.05	0.86	1.01	0.089	0.522	0.597	0.713
	Occludin	0.98	0.91	1.06	1.45	0.125	0.451	0.670	0.991
	ZO-1	0.71	1.34	0.89	1.43	0.143	0.219	0.257	0.108
Ileum	Mucin-2	0.89	0.64	0.99	2.03	0.348	0.482	0.267	0.383
	Occludin	1.00	0.68	0.71	0.82	0.091	0.643	0.557	0.280
	ZO-1	1.13	0.87	1.38	0.45	0.185	0.409	0.394	0.337

<sup>a,b</sup>Mean values with the row with different superscript letter was significant difference ( $P < 0.05$ ).

<sup>1</sup>n = 7 per treatment group; CON, basal diet; EOA, basal diet with encapsulated organic acids and essential oils; SEM, standard error of mean; and ZO-1, zona occludens 1.

**Table 11.** Effects of encapsulated essential oils and organic acids (EOA) on secretory immunoglobulin A (ng/100 mg prot) in intestinal mucosa of laying hens.<sup>1</sup>

Item	CON	EOA (mg/kg)			SEM	P-value		
		150	300	450		ANOVA	Linear	Quadratic
Week 25								
Duodenum	5.31	4.32	5.28	4.59	0.199	0.196	0.495	0.699
Jejunum	5.92	5.52	5.23	5.30	0.173	0.542	0.191	0.513
Ileum	7.56 <sup>a</sup>	7.23 <sup>a,b</sup>	6.19 <sup>b</sup>	6.17 <sup>b</sup>	0.191	0.008	0.001	0.619
Week 30								
Duodenum	6.11	6.01	5.37	5.57	0.21	0.589	0.273	0.730
Jejunum	7.40 <sup>a</sup>	6.70 <sup>a,b</sup>	5.32 <sup>b</sup>	6.05 <sup>a,b</sup>	0.26	0.031	0.018	0.136
Ileum	4.95 <sup>a,b</sup>	5.46 <sup>a</sup>	3.98 <sup>b</sup>	5.18 <sup>a,b</sup>	0.20	0.043	0.611	0.333

<sup>a,b</sup>Mean values with the row with different superscript letter was significant difference ( $P < 0.05$ ).

<sup>1</sup>n = 7 per treatment group; CON, basal diet; EOA, basal diet with encapsulated organic acids and essential oils; SEM, standard error of mean; and sIgA, secretory immunoglobulin A.

*Bifidobacteria* can increase the number of goblet cells in the intestine of chicks and the secretion of mucin-2, and enhance the intestinal mucosal immune function of birds. The secretion of mucus-forming sIgA and mucins reinforces the mucosal barrier on the extraepithelial side (Yang et al., 2019). In our study, the EOA treatments quadratically increased mucin-2 mRNA relative expression (quadratic,  $P < 0.01$ ) in ileum of laying hens at week 25 (Table 10), and it was significantly higher ( $P < 0.05$ ) in birds fed diet contained 300 mg/kg EOA compared to control birds. Meanwhile, there was a linear decrease (linear,  $P < 0.01$ ; ANOVA,  $P < 0.05$ ) in concentration of sIgA in ileum at week 25 and jejunum at week 30 with increasing dietary levels of EOA (Table 11). Secretory immunoglobulin A is the main

antibody to mucosal immunity and can effectively fight infectious diseases and invasion of pathogens into deep tissues (Liu et al., 2015). Consistent with our study results, Sun et al. (2015) reported that thymol and carvacrol lowered the sIgA expression in the ileum. The reason for this phenomenon may be that body resistance is improved after the addition of EOA and the probability of disease occurrence is reduced. Therefore, the body has enough energy for improving the product performance and without needing for synthesis or secretion of much more sIgA to defense the body. On the other hand, the effects of EOA on bacterial cell count and the sIgA expression in the distal intestinal tract still occur because of coating technology.



## CONCLUSIONS

Above all, EOA can act as a promoter for laying hens by improving the performance and egg quality, enhancing the structure and function of intestine, and reducing the risk of disease. Thus, the finding of this study can provide reference information of green feed additives application of EOA in the layers production.

## ACKNOWLEDGMENTS

This work was funded by the National Key Research and Development Program of China (2018YFD0500600) and National Natural Science Foundation of China (31601395 and 31402095), and the Program for Shaanxi Science and Technology (2017ZDXM-NY-087).

## REFERENCES

- Aclkgoz, Z., H. Bayraktar, and O. Altan. 2011. Effects of formic acid administration in the drinking water on performance, intestinal microflora and carcass contamination in male broilers under high ambient temperature. *Asian Australas. J. Anim. Sci.* 24:96–102.
- Adaszynska-Skwrzynska, M., and D. Szczerbinska. 2019. The effect of lavender (*Lavandula angustifolia*) essential oil as a drinking water supplement on the production performance, blood biochemical parameters, and ileal microflora in broiler chickens. *Poult. Sci.* 98:358–365.
- Andreopoulou, M., V. Tsiouris, and I. Georgopoulou. 2014. Effects of organic acids on the gut ecosystem and on the performance of broiler chickens. *J. Hellenic Vet. Med. Soc.* 65:289–302.
- Bottacini, F., D. van Sinderen, and M. Ventura. 2017. Omics of bifidobacteria: research and insights into their health-promoting activities. *Biochem. J.* 474:4137–4152.
- Brenes, A., and E. Roura. 2010. Essential oils in poultry nutrition: main effects and modes of action. *Anim. Feed Sci. Technol.* 158:1–14.
- Broom, L. J. 2015. Organic acids for improving intestinal health of poultry. *Worlds Poult. Sci. J.* 71:630–642.
- Castanon, J. I. 2007. History of the use of antibiotic as growth promoters in European poultry feeds. *Poult. Sci.* 86:2466–2471.
- Donoghue, D. J. 2003. Antibiotic residues in poultry tissues and eggs: human health concerns? *Poult. Sci.* 82:618–621.
- Du, E., W. Wang, L. Gan, Z. Li, S. Guo, and Y. Guo. 2016. Effects of thymol and carvacrol supplementation on intestinal integrity and immune responses of broiler chickens challenged with *Clostridium perfringens*. *J. Animal Sci. Biotechnol.* 7:19.
- Emami, N. K., S. Z. Naeini, and C. A. Ruiz-Feria. 2013. Growth performance, digestibility, immune response and intestinal morphology of male broilers fed phosphorus deficient diets supplemented with microbial phytase and organic acids. *Livest. Sci.* 157:506–513.
- Gheisar, M. M., A. Hosseindoust, and I. H. Kim. 2014. Evaluating the effect of microencapsulated blends of organic acids and essential oils in broiler chickens diet. *J. Appl. Poult. Res.* 24:511–519.
- Habibi, R., G. Sadeghi, and A. Karimi. 2014. Effect of different concentrations of ginger root powder and its essential oil on growth performance, serum metabolites and antioxidant status in broiler chicks under heat stress. *Br. Poult. Sci.* 55:228–237.
- Iqbal, Z., F. Metzger, M. Singh, N. Morgan, R. A. Swick, R. A. Perez-Maldonado, S. A. M'Sadeq, J. Zentek, and I. Ruhnke. 2019. Enzymes and/or combination of organic acid and essential oils supplementation in pasture-fed free-range laying hens increased the digestibility of nutrients and non-starch polysaccharides. *Poult. Sci.* 98:1410–1424.
- Jang, I. S., Y. H. Ko, S. Y. Kang, and C. Y. Lee. 2007. Effect of a commercial essential oil on growth performance, digestive enzyme activity and intestinal microflora population in broiler chickens. *Anim. Feed Sci. Technol.* 134:304–315.
- Jiang, X. R., A. Awati, A. Agazzi, F. Vitari, A. Fearari, H. Bientoet, M. Crestani, C. Domeneghini, and V. Bontempo. 2015. Effects of a blend of essential oils and an enzyme combination on nutrient digestibility, ileum histology and expression of inflammatory mediators in weaned piglets. *Animal.* 9:417–426.
- Kang, H. K., S. B. Park, S. H. Kim, and C. H. Kim. 2016. Effects of stock density on the laying performance, blood parameter, corticosterone, litter quality, gas emission and bone mineral density of laying hens in floor pens. *Poult. Sci.* 95:2764–2770.
- Kaya, A., H. Kaya, M. Gul, Apaydin B. Yildirim, and S. Timurkaan. 2015. Effect of different levels of organic acids in the diets of hens on laying performance, egg quality criteria, blood parameters, and intestinal histomorphology. *Indian. J. Anim. Res.* 49:919–932.
- Khan, S. H., and J. Iqbal. 2016. Recent advances in the role of organic acids in poultry nutrition. *J. Appl. Anim. Res.* 44:359–369.
- Lee, I. K., S. Bae, M. J. Gu, S. J. You, G. Kim, S. M. Park, W. H. Jeung, K. H. Ko, K. J. Cho, J. S. Kang, and C. H. Yun. 2017. H9N2-specific IgG and CD4+CD25+ T cells in broilers fed a diet supplemented with organic acids. *Poult. Sci.* 96:1063–1070.
- Lessire, M., V. Gallo, M. Prato, O. Akide-Ndunge, G. Mandili, P. Marget, P. Arese, and G. Duc. 2017. Effects of faba beans with different concentrations of vicine and convicine on egg production, egg quality and red blood cells in laying hens. *Animal.* 11:1270–1278.
- Liu, L., J. Shen, C. Zhao, X. F. Wang, J. H. Yao, Y. S. Gong, and X. J. Yao. 2015. Dietary astragalus polysaccharide alleviated immunological stress in broilers exposed to lipopolysaccharide. *Int. J. Biol. Macromol.* 72:624–632.
- Liu, Y. L., X. Yang, H. L. Xin, S. Chen, Y. L. Duan, and X. J. Yang. 2017. Effects of a protected inclusion of organic acids and essential oils as antibiotic growth promoter alternative on growth performance, intestinal morphology and gut microflora in broilers. *Anim. Sci. J.* 88:1414–1424.
- Liu, Y., W. Guo, Z. Pu, X. Li, X. Lei, J. Yao, and X. Yang. 2016. Developmental changes of insulin-like growth factors in the liver and muscle of chick embryos. *Poult. Sci.* 95:1396–1402.
- Miska, K. B., R. H. Fetterer, and E. A. Wong. 2014. The mRNA expression of amino acid transporters, aminopeptidase N, and the di- and tri-peptide transporter PepT1 in the embryo of the domesticated chicken (*Gallus gallus*) shows developmental regulation. *Poult. Sci.* 93:2262–2270.
- Mott, C. R., P. B. Siegel, K. E. J. Webb, and E. A. Wong. 2008. Gene expression of nutrient transporters in the small intestine of chickens from lines divergently selected for high or low juvenile body weight. *Poult. Sci.* 87:2215–2224.
- Naghi Shokri, A., H. A. Ghasemi, and K. Taherpour. 2017. Evaluation of aloe vera and synbiotic as antibiotic growth promoter substitutions on performance, gut morphology, immune responses and blood constituents of broiler chickens. *Anim. Sci. J.* 88:306–313.
- Paiva, D., C. Walk, and A. Mcelroy. 2014. Dietary calcium, phosphorus, and phytase effects on bird performance, intestinal morphology, mineral digestibility, and bone ash during a natural necrotic enteritis episode. *Poult. Sci.* 93:2752–2762.
- Schwartz, A., M. Jacobi, J. S. Frick, M. Richter, K. Rusch, and H. Kohler. 2010. Microbiota in pediatric inflammatory bowel disease. *J. Pediatr.* 157:240–244.e1.
- Senkoylu, N., H. E. Samli, M. Kanter, and A. Agma. 2007. Influence of a combination of formic and propionic acids added to wheat- and barley-based diets on the performance and gut histomorphology of broiler chickens. *Acta. Vet. Hung.* 55:479–490.
- Speier, J. S., L. Yadgary, Z. Uni, and E. A. Wong. 2012. Gene expression of nutrient transporters and digestive enzymes in the yolk sac membrane and small intestine of the developing embryonic chick. *Poult. Sci.* 91:1941–1949.
- Su, S., K. B. Miska, R. H. Fetterer, M. C. Jenkins, and E. A. Wong. 2014. Expression of digestive enzymes and nutrient transporters in *Eimeria acervulina*-challenged layers and broilers. *Poult. Sci.* 93:1217–1226.

- Taverniers, I., P. Windels, M. Vaïtilingom, A. Milcamps, B. E. Van, D. E. G. Van, and D. L. Marc. 2005. Event-specific plasmid standards and real-time PCR methods for transgenic Bt11, Bt176, and GA21 maize and transgenic GT73 canola. *J. Agric. Food Chem.* 53:3041–3052.
- Walk, C. L., M. R. Bedford, and A. P. Mcelroy. 2012. Influence of limestone and phytase on broiler performance, gastrointestinal pH, and apparent ileal nutrient digestibility. *Poult. Sci.* 91:1371–1378.
- Wu, Y., Y. Zhou, C. Lu, H. Ahmad, H. Zhang, J. He, L. Zhang, and T. Wang. 2016. Influence of butyrate loaded clinoptilolite dietary supplementation on growth performance, development of intestine and antioxidant capacity in broiler chickens. *PLoS One.* 11:e0154410.
- Yang, X., Y. Liu, F. Yan, C. Yang, and X. Yang. 2019. Effects of encapsulated organic acids and essential oils on intestinal barrier, microbial count, and bacterial metabolites in broiler chickens. *Poult. Sci.* 98:2858–2865. doi:10.3382/ps/pez031.
- Yin, D., E. Du, J. Yuan, J. Gao, Y. Wang, S. E. Aggrey, and Y. Guo. 2017. Supplemental thymol and carvacrol increases ileum *Lactobacillus* population and reduces effect of necrotic enteritis caused by *Clostridium perfringens* in chickens. *Sci. Rep.* 7:7334.
- Yu, C., J. Wei, C. Yang, Z. Yang, W. Yang, and S. Jiang. 2018. Effects of star anise (*Illicium verum* Hook.f.) essential oil on laying performance and antioxidant status of laying hens. *Poult. Sci.* 97:3957–3966.
- Yuan, J., B. Zhang, and Y. Guo. 2012. Poultry fat decreased fatty acid transporter protein mRNA expression and affected fatty acid composition in chickens. *J. Anim. Sci. Technol.* 3:17–22.
- Zeng, Z. k., S. Zhang, H. L. Wang, and X. S. Piao. 2015. Essential oil and aromatic plants as feed additives in non-ruminant nutrition: a review. *J. Anim. Sci. Technol.* 6:7–16.