# The Regulatory Effects of The Combined Dietary Supplementation of Essential Oils And Organic Acids On The Microbial Communities of Cobb Broilers.

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**Research**

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# Abstract

**Background:** The emergence and spread of antibiotic resistance genes in pathogenic microorganisms have led to many countries enacting restricted use of antibiotics as growth promoters in animal feed. The combined use of essential oils and organic acids can help maintain intestinal health, improve animal growth performance, and alleviate the negative effects of banned antibiotics for certain economically important animals. However, the modes of action for the combined dietary supplementation of essential oils and organic acids (thymol-citric acid; EOA1, and thymol-butyric acid; EOA2) remain unclear, although it is speculated that their activities are achieved through beneficial modulation of gastrointestinal microbial communities and the inhibition of pathogen growth. In this study, 16S rDNA amplicon sequencing was used to analyze the effects of treatment with EOA1 and EOA2 on the jejunal, cecal, and fecal microbial communities of Cobb broilers (using enramycin and virginiamycin as positive controls) while also evaluating effects over different broiler ages (14, 28, 35, and 42 days old).

**Results:** We found that the intestinal microbial communities of the broilers developed with increasing age, while *Lactobacillus* gradually came to dominate intestinal communities. Further, the microbial communities of feces were more complex than in the jejuna and ceca. The longitudinal changes in these communities were systematically explored for broilers of different ages. The addition of EOA1 or EOA2 to the diet: 1) inhibited the proliferation of *Ralstonia pickettii* and Alcaligenaceae in jejuna on the 28th day, 2) promoted the colonization and growth of beneficial bacteria like *Lactobacillus*, Clostridia, and Bacteroidia at various growth stages, and 3) enriched the abundances of certain microbiota functions including biological pathways related to metabolism (e.g., enzyme families).

**Conclusions:** EOA1 and EOA2 dietary supplementation can affect various microbial metabolic pathways related to the metabolism and absorption of nutrients via the regulation of intestinal microbial community structures of Cobb broilers, while also playing an important role in promoting host growth.


# Background

Antibiotics, including especially penicillin, tetracycline, and streptomycin can prevent and treat potentially pathogenic bacterial infections and reduce morbidity and mortality from infections by affecting intestinal microbial community composition and the colonization of the intestinal tract by pathogenic bacteria when used in livestock and poultry at subtherapeutic levels [1–3]. However, antibiotics do not selectively affect the colonization of intestines by pathogenic bacteria and can interfere with other members of intestinal microbial communities [4], The overuse of antibiotics causes environmental pollution in air, soils, and waters, but it also poses risks to human health through foodborne antibiotic residues and selective resistance to antibiotics in some pathogens [5]. In addition, the long-term use of antibiotics can create selective pressures, resulting in disruption of intestinal microbial communities and promoting the development of resistance in pathogenic bacteria. These antibiotic-resistant strains can carry antibiotic resistance genes enabling increased ability to resist antibiotic treatment and increasing the risk of

infection or re-infection [2, 6]. Therefore, it is urgent to stop antibiotic use and develop better antibiotic substitutes for use in animal feeds.

The application of essential oils and organic acids in animal husbandry has gradually increased in recent years. Organic acids that exhibit significant dissociation potentials, including lactic acid, citric acid, and fumaric acid, have been reported to inhibit and sterilize bacteria by directly acting on them in the gastrointestinal tract by lowering the pH and preventing or inhibiting the proliferation of acid-sensitive bacteria [7]. In contrast, organic acids with low dissociation potentials, such as formic acid, butyric acid, and acetic acid, can freely penetrate bacterial cell membranes and enter cells, thereby lowering intracellular pH, disrupting normal cellular functions, such as bacterial DNA replication and protein synthesis, and inhibiting the proliferation of pathogens [8]. These activities indirectly reduce the competition between intestinal microbiota and hosts, while improving intestinal digestibility [9]. In addition, organic acids can improve gastrointestinal tract morphology, promote the secretion of trypsin and chymotrypsin from the pancreas, improve proteolytic enzyme activity, improve nutrient digestibility, and improve growth performance [7, 10]. Essential oils are effective antimicrobial agents that can increase feed palatability, stimulate intestinal mucus secretion, prevent pathogenic bacteria from adhering to intestinal epithelia, improve intestinal morphology, maintain intestinal microbiota, regulate immune responses, reduce oxidative stress, and improve growth performance [11, 12]. Within common essential oils, thymol is the primary component.

However, the use of a single feed additive still has some drawbacks when selecting alternative antibiotics. For example, single additives are not effective enough to address the complex and changing environments of livestock and poultry production environment. Thus, the combined use of different feed additives has become a focus of current research. Organic acids can complement essential oil use by achieving synergistic effects to mediate antibacterial and bactericidal activities [13]. For example, diets supplemented with thymol, fumaric acid, and sorbic acid can maintain intestinal morphology, significantly reduce the abundances of harmful bacteria (e.g., *Escherichia coli*), increase the concentrations of short chain fatty acids (SCFAs), increase digestive enzyme activity, and promote digestive and absorptive capacity, in addition to increasing intestinal barrier function in laying hens [14], or broilers [15]. Further, thymol and benzoic acid complexes in conjunction with cinnamaldehyde and caproic acid complexes inhibit the proliferation of *Salmonella* [16]. Thus, the combined use of essential oils and organic acids can improve animal health, and their modes of action are closely related to intestinal microbiota dynamics. Our previous study observed that diets supplemented with thymol-citric acid (EOA1) or thymol-butyric acid (EOA2) positively affected Cobb broiler health, with similar efficacy as antibiotics. However, nothing is known of the effects of EOA1 or EOA2 on the intestinal microbiota of Cobb broilers. Therefore, this study analyzed the effects of age (14, 28, 35, and 42 days old) on changes in intestinal (jejunal, cecal, and fecal) microbial communities in Cobb broilers, while also using enramycin (EM) and virginiamycin (VM) to investigate the regulatory effects of Cobb broiler intestinal microbial communities by EOA1 and EOA2. The results of this study provide a theoretical basis for the application of these oils as alternatives to antibiotics in poultry diets.

# Methods

## Animals, diets, and experimental designs

A total of 1,680 one-day-old Cobb broilers exhibiting good health and similar body weights were randomly divided into 5 groups with 12 replicates per group and 28 Cobb broilers per replicate using a single-factor experimental design. The groups were constructed with feeding of: 1) a basal diet (control group), 2) a basal diet + 20 mg/kg virginiamycin (VM group), 3) a basal diet + 10 mg/kg enramycin (EM group), 4) a basal diet + 150 mg/kg thymol + 2 g/kg citric acid (EOA1 group), and 5) a basal diet + 150 mg/kg thymol + 2 g/kg butyric acid (EOA2 group). The experiments were conducted over 42 days. The basic diets and nutritional compositions are shown in (Table S1).

The experiment was conducted at a broiler factory in Hangu, Tianjin. The chicken house was rigorously cleaned and disinfected before the experiment. During the experiment, all of the Cobb broilers were freely allowed to drink and feed. The temperature of the chicken house was controlled at about 33°C for the $1^{st}$–$7^{th}$ days of the experiment, followed by a gradual reduction to 23°C on the $7^{th}$–$21^{st}$ days, and then maintained at 23°C thereafter. Light was provided 24 h per day for the $1^{st}$–$17^{th}$ days, and 16 h per day thereafter. The house was well ventilated and routine immunization was conducted regularly according to standard protocols.

## Sample collection and processing

On days 1 (before feeding), 14, 28, 35, and 42, the feces from six healthy, randomly selected Cobb broilers with similar body weights were collected and stored at -80°C until later DNA extraction. The Cobb broilers were also sacrificed by cervical dislocation on days 14, 28, 35, and 42 after fasting for 12 h before dissection (although they were allowed to drink freely). The contents of the jejuna and ceca were aseptically collected and stored at -80°C for later genomic DNA extraction.

## 16S rDNA amplicon sequencing

After the samples were thawed, the genomic DNA of the Cobb broiler intestinal contents was extracted using the cetyltrimethyl ammonium bromide (CTAB) method. The concentrations and purity of DNA were evaluated with agarose gel electrophoresis, followed by dilution of DNA to 1 ng/μL with sterile water, and storage at -80°C for subsequent experiments. The 16S rRNA gene V3-V4 regions were amplified with the primers 341F: 5'-CCTAYGGGRBGCASCAG and 806R: 5'-GGACTACNNGGGTATCTAAT-3' with barcodes attached, and with Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs, Ipswich, MA) and high-fidelity enzymes. The purity and concentrations of PCR products were detected with 2% agarose gel electrophoresis and the PCR products of target bands were recovered with a Qiagen gel recovery kit (Qiagen, Hilden, Germany). The TruSeq® DNA PCR-Free sample preparation kit was used to construct an amplicon library. After the samples were assessed for quality, they were sequenced on an Illumina NovaSeq6000 platform (Nuohe Zhiyuan Biological Information Technology Co., Ltd., Beijing, China).

### Statistical analyses

The Flash v.1.2.7 software program [17], was used to splice PE reads after removing primer and barcode sequences, thereby obtaining raw sequence tags. The Qiime v.1.9.1 software program was then used to filter sequences to obtain high-quality clean tags. The Qiime software program (v.1.9.1) pipeline [18], was used for the quality control of sequence tags. Chimeric sequences were removed by comparing sequences against a database, as previously described [19]. The Uparse V7.0.1001 software program [20], was used to cluster clean sequence tags and those with over 97% nucleotide identity were classified into the same operational taxonomic units (OTUs). The classification method of Mothur using the SSUrRNA database [21], of SILVA132 [22], was used to annotate and classify representative OTU sequences.

Shannon and Coverage indices were calculated using the Qiime software program, while one-way ANOVA tests were conducted in SPSS 22.0 to investigate differences in the diversity of Cobb broiler intestinal microbial communities. OTU abundance information was used to construct histograms of species composition, conduct principal coordinates analysis (PCoA) in R (Version 2.15.3), and conduct linear discriminant analysis effect size (LEfSe) analyses based on linear discriminant analysis (LDA) distribution histograms and LEfSe cladograms. The default LDA score of 4.0 was used for the tests. The combined analyses were used to directly compare differences in microbial community composition among broiler intestines. Metabolic functions of microbial communities were predicted using Tax4fun [23], and a cluster heat map of Tax4fun functional annotation was drawn at level 2 to reveal the effects of EOA1 and EOA2 on Cobb broiler intestinal microbial functions.

## Results

# Changes in the intestinal microbiota of Cobb broilers at different growth stages

The 16S rDNA amplicon sequencing was used to analyze the intestinal microbiota within the feces, jejuna, and ceca of Cobb broilers at different growth stages. After merging quality-filtered reads obtained from Illumina NovaSeq sequencing, a total of 2,254,549 effective sequence tags (median: 59,722 per sample; range of 36,084–69,463) were retained. (Fig. 1a-c) shows the longitudinal changes in intestinal microbial community diversity within Cobb broilers of different ages. The one-day-old Cobb broilers exhibited significantly lower fecal microbial diversity than those from other ages, reflecting an initial state of intestinal microbial community establishment. Feces exhibited high microbial diversity on day 14, but sharply decreased on days 28 and 35, followed by another increase on day 42. The jejunal communities exhibited relatively high microbial diversity on day 14, but decreased diversity on days 28, 35, and 42. Ceca communities exhibited similar microbial diversity levels on days 14 and 35 that were lower than those observed on days 28 and 42. The above results indicate longitudinal changes in microbial community diversity within the three intestinal components analyzed here.

PCoA analysis of Bray-Curtis distances (Fig. 1d-f) revealed significant differences in the intestinal microbial community structures of Cobb broilers at different growth stages. In particular, the fecal microbiota compositions of 42-day-old Cobb broilers were significantly different from those at other ages. The jejunal microbiota compositions were significantly different between communities in the 14–28- and 35–42-day-old broilers. Further, the cecal microbiota communities exhibited differences across the four growth stages. The 10 most abundant phyla and genera were used to investigate differences in the means of taxonomic relative abundances within the fecal, jejunal, and cecal microflora among the 5 different treatment groups (Fig. 2). At the phylum level, Firmicutes were dominant in the broiler intestinal microflora, followed by the Proteobacteria that accounted for relatively high proportions of fecal and jejunal communities, and the Bacteroidetes that accounted for relatively high proportions of cecal microflora. However, the abundances of the above taxa exhibited significant differences among different broiler growth stages. Firmicutes abundances in feces and ceca slowly increased from day 14 to 42. In addition, the *Lactobacillus* genus accounted for a relatively minor proportion of the feces of 1-day-old broilers, which significantly differed from their abundances at other time points. In contrast, *Lactobacillus* was the dominant genus in broiler feces and jejuna from 14 to 42 days, while they were completely dominant in ceca only at day 35. Further, *Faecalibacterium*, *Alistipes*, and *Bacteroides* accounted for higher proportions of cecal communities, although their abundances were significantly different among different broiler growth stages.

# Distinct microflora members within broilers of different growth stages

An LDA score of 4.0 was used for LEfSe analysis at the phylum to species taxonomic levels in order to determine specific populations associated with broilers at different growth stages (Fig. 3). A total of 29 significantly different representative taxa were identified in the feces. Among them, 10 species, including *Enterococcus faecium*, *Sphingobacterium mizutaii*, and *Lactobacillus amylotrophicus*, were significantly enriched in 1-day-old broiler feces. Ten microbial taxa were also significantly enriched in 14-day-old Cobb feces, including *Ralstonia pickettii*, *Klebsiella pneumoniae*, and Lachnospiraceae. Only *Lactobacillus johnsonii* was significantly enriched in 28-day-old broiler feces. *Lactobacillus salivarius* was significantly enriched in 35-day-old broiler feces and significant enrichment of a single lineage in 42-day-old broiler feces was observed for a lineage associated with the Firmicutes, Bacilli, Lactobacillales, Lactobacillaceae, *Lactobacillus*, and *Lactobacillus aviarius*. A total of 12 significantly differential taxa were observed for the jejunal communities including the Enterobacterales and *Lactobacillus reuteri* that were significantly enriched in 14-day-old broiler jejunal samples. Significant enrichment was observed for 1 lineage of organisms in the 28-day-old broiler jejunal communities comprising Gammaproteobacteria, Burkholderiales, Burkholderiaceae, *Ralstonia*, and *Ralstonia pickettii*. In addition, *Acinetobacter radioresistens* abundances were significantly enriched in 35-day-old broiler jejunal communities. The taxa *Lactobacillus aviaries*, *Alistipes onderdonkii*, *Barnesiella*, and DTU089 were significantly enriched in 42-day-old broiler jejunum communities. In the cecal communities, 34 significantly differential representative

taxa were identified. Among these, Bacteroidales and Clostridia were significantly enriched in 14-day-old broiler ceca. In addition, six microbial taxa, including *Alistipes inops*, *Bacteroides fragilis*, and Oscillospiraceae, were significantly enriched in 28-day-old broiler ceca. Further, 3 lineages were significantly enriched in 35-day-old broiler ceca, namely: (1) Bacilli, Lactobacillales, Lactobacillaceae, *Lactobacillus*, and *Lactobacillus aviaries*, (2) Anaerolineae and unidentified *Anaerolineae*, and (3) *Barnesiella*. Likewise, 2 lineages were significantly enriched in 42-day-old broiler ceca, namely: (1) Tannerellaceae, *Parabacteroides*, and *Parabacteroides* sp. CT06; and (2) Clostridia, Oscillospirales, Ruminococcaceae, and *Faecalibacterium*. The above results demonstrated that the intestinal microbial communities of Cobb broilers developed with age.

To further investigate differences of microbial communities among different intestinal sites, the three components (jejuna, ceca, and feces) of 42-day-old broilers were used for LEfSe analysis with an LDA threshold score of 4.0 (Fig. 4). There were relatively greater representative bacteria in feces, with 19 taxa (e.g., Bacteroidales, Clostridia, and *Helicobacter*) significantly enriched in those communities. The jejuna exhibited the next most numbers of representative taxa with three lineages that were significantly enriched including (1) Gammaproteobacteria, Enterobacterales, Enterobacteriaceae, *Escherichia-Shigella*, and *Escherichia coli*, (2) *Lactobacillus johnsonii*, and (3) *Lactobacillus reuteri*. Only one lineage of taxa was significantly enriched in the ceca: Bacilli, Lactobacillales, Lactobacillaceae, *Lactobacillus*, and *Lactobacillus aviarius*.

# Regulatory effects of EOA1 and EOA2 on Cobb broiler intestinal microbiota

We previously observed that diets supplemented with EOA1 or EOA2 could improve broiler growth performance, with effects similar to the use of antibiotics Table S2. In this study, we investigated changes in intestinal microbial community diversity of Cobb broilers at different time points after supplementing diets with EOA1 and EOA2. A total of 10,474,315 effective high-quality sequence tags (36,084–69,576 per sample) were obtained for subsequent analysis. -diversity indices Table 1 of fecal, jejunal, and cecal communities were analyzed for each group of broilers at 14, 28, 35, and 42 days of age. The coverage index estimates for the broiler feces, jejunal, and cecal communities at different growth stages in each group were nearly 99%, indicating that the sequencing depth used here was adequate to detect native diversity within the samples. At 14 days of age, the fecal community Shannon index values were significantly lower in the EOA1 group than in the control and VM groups ($p < 0.05$). The cecum community Shannon index values were significantly higher in the EM group than in the other four groups ($p < 0.05$), while the cecum Shannon index values were significantly lower in the control group than in the other four groups ($p < 0.05$). At 28 days of age, the fecal community Shannon index values in the EM group were lower than in the EOA2 group ($p < 0.05$). In addition, the jejunal community Shannon index values of the control, VM, and EOA2 groups were significantly lower than in the EOA1 group ($p < 0.05$). Further, the cecum community Shannon index values in the EOA1 and EOA2 groups were significantly higher than in the other three groups ($p < 0.05$), while the cecum community Shannon index values in the VM group were

lower than in the control and EM groups ($p < 0.05$). In broilers at 35 days of age, the fecal community Shannon index values were significantly higher in the EOA2 group than in the control group ($p < 0.05$). In contrast, the jejunal community Shannon index values were significantly lower in the control and VM groups compared to the EM and EOA1 groups ($p < 0.05$), while the cecum community Shannon index values were significantly higher in the EOA2 group than in the VM and EOA1 groups ($p < 0.05$). Considering the 42-day-old broilers, the lowest fecal community Shannon index value was observed for the EM group. These results indicate that diets supplemented with EOA1 or EOA2 can significantly alter the cecal, jejunal, and fecal microbial community diversity in Cobb broilers, while these effects are also influenced by broiler age.

Table 1

Effect of different treatment groups on the intestinal microbial alpha diversity index of Broilers[1].

| Items | | Groups | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Control | VM | EM | EOA1 | EOA2 | SEM[2] | *P* |
| **Day 14** | | | | | | | | |
| Feces | Shannon | 4.50$^{a}$ | 4.35$^{a}$ | 4.05$^{ab}$ | 2.79$^{b}$ | 3.90$^{ab}$ | 0.24 | 0.149 |
| | Goods coverage | 0.9930$^{b}$ | 0.9943$^{ab}$ | 0.9933$^{ab}$ | 0.9980$^{a}$ | 0.9980$^{a}$ | 0.0008 | 0.077 |
| Jejunum | Shannon | 2.01 | 1.67 | 2.16 | 2.24 | 2.21 | 0.18 | 0.888 |
| | Goods coverage | 0.9983 | 0.9980 | 0.9977 | 0.9973 | 0.9983 | 0.0003 | 0.772 |
| Cecum | Shannon | 5.16$^{c}$ | 6.01$^{b}$ | 6.55$^{a}$ | 5.73$^{b}$ | 6.03$^{b}$ | 0.13 | 0.001 |
| | Goods coverage | 0.9980$^{a}$ | 0.9980$^{a}$ | 0.9933$^{b}$ | 0.9980$^{a}$ | 0.9980$^{a}$ | 0.0006 | 0.019 |
| **Day 28** | | | | | | | | |
| Feces | Shannon | 2.74$^{ab}$ | 2.78$^{ab}$ | 2.25$^{b}$ | 2.45$^{ab}$ | 2.88$^{a}$ | 0.09 | 0.110 |
| | Goods coverage | 0.9977 | 0.9980 | 0.9983 | 0.9980 | 0.9980 | 0.0001 | 0.351 |
| Jejunum | Shannon | 2.19$^{b}$ | 1.64$^{c}$ | 2.35$^{ab}$ | 2.50$^{a}$ | 2.06$^{b}$ | 0.09 | 0.001 |
| | Goods coverage | 0.9990 | 0.9990 | 0.9987 | 0.9990 | 0.9993 | 0.0001 | 0.351 |
| Cecum | Shannon | 6.43$^{b}$ | 5.75$^{c}$ | 6.63$^{b}$ | 7.26$^{a}$ | 7.18$^{a}$ | 0.16 | 0.000 |
| | Goods coverage | 0.9980$^{a}$ | 0.9980$^{a}$ | 0.9947$^{b}$ | 0.9933$^{b}$ | 0.9920$^{b}$ | 0.0008 | 0.004 |
| **Day 35** | | | | | | | | |
| Feces | Shannon | 2.27$^{b}$ | 2.54$^{ab}$ | 2.73$^{ab}$ | 2.71$^{ab}$ | 3.00$^{a}$ | 0.09 | 0.050 |
| | Goods coverage | 0.9980 | 0.9973 | 0.9980 | 0.9980 | 0.9983 | 0.0002 | 0.785 |
| Jejunum | Shannon | 1.70$^{b}$ | 1.65$^{b}$ | 2.49$^{a}$ | 2.29$^{a}$ | 2.04$^{ab}$ | 0.11 | 0.013 |
| | Goods coverage | 0.9980 | 0.9980 | 0.9987 | 0.9987 | 0.9990 | 0.0002 | 0.205 |
| Cecum | Shannon | 5.26$^{abc}$ | 4.67$^{c}$ | 5.66$^{ab}$ | 5.07$^{bc}$ | 5.92$^{a}$ | 0.15 | 0.021 |

| Items | | Groups | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Control | VM | EM | EOA1 | EOA2 | SEM[2] | *P* |
| | Goods coverage | 0.9970[a] | 0.9970[a] | 0.9970[a] | 0.9920[ab] | 0.9873[b] | 0.0013 | 0.024 |
| **Day 42** | | | | | | | | |
| Feces | Shannon | 3.30[ab] | 3.98[a] | 2.86[b] | 3.69[a] | 3.39[ab] | 0.13 | 0.047 |
| | Goods coverage | 0.9977[a] | 0.9977[a] | 0.9980[a] | 0.9927[b] | 0.9933[b] | 0.0008 | 0.019 |
| Jejunum | Shannon | 1.02 | 1.34 | 1.20 | 1.47 | 1.56 | 0.08 | 0.247 |
| | Goods coverage | 0.9990 | 0.9990 | 0.9990 | 0.9990 | 0.9990 | 0.0000 | |
| Cecum | Shannon | 6.23 | 5.92 | 6.44 | 6.31 | 6.95 | 0.17 | 0.456 |
| | Goods coverage | 0.9947 | 0.9973 | 0.9980 | 0.9973 | 0.9923 | 0.0009 | 0.284 |

[1]Value with different small letters mean significant difference ($P < 0.05$).

[2]SEM, standard error of means.

To identify specific intestinal taxa within broilers of different treatment groups, LEfSe analysis was used to compare communities among groups. At 14 days of age, 11 microbial taxa exhibited significantly different abundances in the feces of groups, with one microbial taxon significantly associated with the jejunum and 38 microbial taxa that were significantly associated with the ceca (Fig. 5a, 6a, 7a). After supplementing diets with VM, 14-day-old broilers exhibited significantly higher abundances of *Enterococcus faecium*, *Aerococcus*, and Aerococcaceae in feces, while *Lactobacillus reuteri* abundances in the jejuna were significantly lower. Significantly enriched flora in the ceca were associated with Clostridia (primarily including Lachnospiraceae, *Oscillibacter*, Butyricicoccaceae, Ruminococcaceter, and the [Eubacterium] coprostanoligenes group). Five microbial taxa were significantly enriched in the ceca of 14-day-old broilers in the EM group, including *Faecalibacterium prausnitzii*, *Bifidobacterium*, and Actinobacteriota. When EOA1 group broilers were 14 days old, Enterococcaceae, *Enterococcus*, and *Enterococcus cecorum* were significantly enriched in feces, and significantly enriched taxa in the ceca were associated with the Bacilli (including the Clostridia vadinBB60, UCG__005, and Ruminococcaceae groups). *Lactobacillus aviarius* and *Lactobacillus johnsonii* were significantly enriched in the feces of 14-day-old broilers in the EOA2 group, while 10 taxa were significantly enriched in the cecal communities including *E. coli*, Oscillospiraceae, and *Faecalibacterium*. Thirteen microbial taxa were significantly enriched in the feces of 28-day-old broilers, while 17 were significantly enriched in jejuna, and 32 were significantly enriched in ceca (Fig. 5b, 6c, 7b). In the control group, 4 taxa (e.g., *Lactobacillus* phage Sal3 and *Lactobacillus salivarius*) were significantly enriched in the feces of 28-day-old broilers, and 8 taxa

(e.g., *Lactobacillus reuteri*, *Ralstonia pickettii*, *Lactobacillus salivarius*, Campylobacteria, and Alcaligenaceae) were significantly enriched in the jejuna, and 5 taxa (e.g., Oscillospirales and *Alistipes*) were enriched in the ceca.

After diet supplementation with VM, the abundances of fecal *Gordonibacter* were significantly higher in 28-day-old broilers, while *Lactobacillus aviarius* was significantly enriched in jejunal communities, and four lineages were significantly enriched in the ceca, including (1) *Alistipes* sp. CHKCI003, (2) Lachnospirales, Lachnospiraceae, (3) Bacteroidota, Bacteroidia, Bacteroidales, Bacteroidaceae, *Bacteroides*, *Bacteroides dorei*, and (4) Lactobacillales, Lactobacillaceae, *Lactobacillus*. After diet supplementation with EM, 28-day-old broilers exhibited significant enrichment of *Clostridium* sp. AUH-JLC140, *Lactobacillus aviaries*, and unidentified *Oscillospiraceae* in the feces. In addition, seven taxa (e.g., *Lolium perenne* and *Dietzia maris*) were significantly enriched in jejunal communities, while Bacteroidaceae, Bacteroides, and *Bacteroides fragilis* were significantly enriched in cecal communities. Tannerellaceae, *Parabacteroides*, *Parabacteroides merdae*, RF39, and Proteobacteria were more abundant in the ceca of 28-day-old broilers in the EOA1 group. When broilers in the EOA2 group were 28 days old, five microbial taxa (e.g., *Enterococcus*, *Kurthia* sp. 11kri321 and Corynebacteriaceae) were significantly enriched in feces, while only *Lactobacillus johnsonii* was significantly enriched in jejuna, and seven microbial taxa (e.g., *Alistipes inops*, *Coprobacter*, and *Rikenella microfusus*) were significantly enriched in ceca. At 35 days of age, 2 microbial taxa were significantly enriched in the fecal and jejunal communities, while 14 microbial taxa were significantly enriched in the ceca (Figure. 5c, 6b, 7c). Four microbial taxa (e.g., *Lactobacillus aviarius*, *Barnesiella*, and the Clostridia vadinBB60 group) were significantly enriched in the ceca of the 35-day-old control group broilers. When broilers with VM supplemented in the diet were 35 days old, *Lactobacillus salivarius* was significantly enriched in the feces and *Lactobacillus aviarius* was significantly enriched in jejuna. After diet supplementation with EM in 35-day-old broilers, 5 microbial taxa (e.g., Oscillospiraceae, Gammaproteobacteria, and Enterobacteriaceae) were significantly enriched in their ceca. Likewise, 35-day-old broilers with diets supplemented with EOA1 exhibited significant enrichment of *Lactobacillus salivarius* in jejuna, in addition to significant enrichment of *Parabacterioides merdae* and *Bacterium* ic1379 in their ceca. Moreover, 35-day-old broilers with EOA2 diet supplementation exhibited significant enrichment of *Lactobacillus aviarius* in feces, but significant enrichment of Butyricicoccaceae, Butyricicoccus, and *Butyricicoccus pullicaecorum* in the ceca. No significantly enriched species were observed in 42-day-old broiler feces, while 13 taxa were significantly enriched in the jejuna, and 29 were significantly enriched in the ceca (Fig. 6d, 7d). Four microbial taxa (e.g., Oscillospirales, *Faecalibacterium*, and *Parabacteroides_sp_CT06*) were significantly enriched in the ceca of 42-day-old control broilers. In 42-day-old broilers with diet supplementation of VM, *Lactobacillus salivarius* were significantly enriched in the jejuna, and 13 microbial taxa (e.g., *Alistipes* sp. CHKCI003, *Alistipes onderdonkii*, *Parabacteroides merdae*, *Rikenella*, and *Barnesiella*) were significantly enriched in the ceca. In 42-day-old broilers with diet supplementation of EM, six microbial taxa (e.g. bacterium ic1277 and unidentified chloroplasts) were significantly enriched in jejuna, while *Alistipes inops*, *Bacteroides dorei*, and the Clostridia vadinBB60 groups were significantly enriched in the ceca. Forty-two day old EOA1 group broilers exhibited significant enrichment of one lineage (Firmicutes, Bacilli, Lactobacillales,

Lactobacillaceae, *Lactobacillus*, and *Lactobacillus johnsonii*) in the jejuna, while the *[Ruminococcus] torques* group, Gammaproteobacteria, and Proteobacteria were significantly enriched in their ceca. Six microbial taxa were significantly enriched in the 42-day-old EOA2 broiler ceca including *Lactobacillus*, *Lactobacillus aviaries*, and Pseudomonadales.

To understand the effects of EOA1 and EOA2 dietary supplements on intestinal microbial community function, functional annotation was performed for the fecal, jejunal, and cecal communities for 42-day-old broilers. The 35 most microbial abundant functions were evaluated using level 2 annotations (Fig. 8). Diets supplemented with VM led to lower inferred abundances of functions involved in the categories of xenobiotic biodegradation and metabolism, transcription, membrane transport, cellular community prokaryotes, and signal transduction. In contrast, several functional categories were enriched including glycan biosynthesis and metabolism, transport and catabolism, biosynthesis of other secondary metabolites, and other biological processes in the ceca. Diets supplemented with EM led to lesser effects on fecal and cecal biological processes, but enhanced biological processes in the jejuna related to metabolism (e.g., metabolism of terpenoids and polyketides, energy metabolism, and metabolism of cofactors and vitamins). In contrast, these treatments were associated with decreased inferred biological process functions related to cellular processes, genetic information processing, and environmental information processing. Diets supplemented with EOA1 or EOA2 resulted in less enrichment of jejunal and cecal biological process functions, but increased abundances of functions involved in fecal lipid metabolism, enzyme families, and xenobiotics biodegradation and metabolism, but decreased enrichment of functions involved in nucleotide metabolism and replication and repair. Thus, EOA1 and EOA2 treatment generally enhanced certain biological pathways related to metabolism, but led to decreased abundances of inferred functions related to cellular processes, genetic information processing, and environmental information processing.

# Discussion

Intestinal microbiota are dynamic and highly complex ecosystems. Interactions of these populations with hosts lead to key roles in tissue and organ morphological development, immune and metabolic processes, and the overall health of animal bodies [24, 25]. Factors including host diets [26], vages [27, 28], antibiotic use [29], or pathogenic infections [30], can cause rapid and continuous changes in body physiological parameters, thereby leading to changes in intestinal microbial community structures. Changes in microbial community compositions or in individual microbial genomes can then lead to changes in overall intestinal microbiomes, thereby affecting transcriptional, translational and metabolic processes [24]. Based on these observations, this experiment investigated the effects of different Cobb broiler growth stages and the combined use of essential oils and organic acids (EOA1 and EOA2) on jejunal, cecal, and fecal microbial communities in order to better understand the potential applications of EOA1 and EOA2 in broiler diets.

Microbial colonization of host intestines begins at birth and their compositions change with host development [24]. In this study, we analyzed changes in the jejunal, cecal, and fecal microbial

communities of Cobb broilers at different growth stages. The microbial communities in all three components of the intestines exhibited higher community diversity 14 days after birth, suggesting a high degree of competition for nutrient and ecological niche resources in the gastrointestinal tract during this period that likely leads to highly dynamic compositional changes [31]. Firmicutes were the dominant phylum in the intestines of Cobb broilers across different growth stages, which is consistent with previous studies [32]. Firmicutes comprise beneficial bacteria (e.g., *Clostridium scindens*, *Clostridium* cluster IV–XIVa) as well as pathogenic bacteria (e.g., *Clostridium difficile*, Streptococci, and Enterococci) that are all components of normal intestinal microbiota [33]. The abundances of intestinal pathogenic bacteria are maintained at low levels, but increase in abundance when the host immune system is disturbed or bacteria migrate outward due to increased intestinal permeability, thereby leading to intestinal disease [34]. In addition, Proteobacteria accounted for a higher proportion of broiler fecal and jejunal microbiota, and their relative abundances decreased with age. This result could be due to rapid changes in the intestinal environment of young broilers, leading to unstable internal microbial intestinal structures that lack diversity. Such characteristics could provide opportunities for food-borne pathogens to colonize intestines. In contrast, increased diversity that arises with age leads to gradual maturation of the intestinal spatial environment that promotes the colonization of commensal bacteria and maintains a dynamic balance of intestinal microbiota.[35] Bacteroidetes are gram-negative bacteria that are extremely well adapted to intestinal environments, wherein they are able to ferment indigestible carbohydrates and produce SCFAs [33]. Bacteroidetes accounted for a relatively high proportion of cecal microbiota communities, which is consistent with the cecum being the main site for microbial fermentation within hosts [36]. *Lactobacillus* was the dominant genus in the fecal and jejunal microbiota and a series of strains or species successively colonized the intestines with broiler age. It is worth noting that lactic acid produced by *Lactobacillus* can be converted to SCFAs that can negatively regulate the NF-κB signaling pathway used to maintain immune cell homeostasis and intestinal health [37–40]. Broiler fecal microbial communities are generally similar to intestinal microbial communities, such that fecal microbial communities can reflect the overall microbial community characteristics of small intestines and ceca to some extent [41]. These similarities may reflect the enrichment of diverse microbial flora in feces. The main function of the jejunum is to absorb nutrients and support the growth of gram-positive and parthenogenic anaerobic bacteria [42]. explaining why the Enterobacteriaceae, *Lactobacillus johnsonii*, and *Lactobacillus reuteri* were significantly enriched in jejuna. Overall, *Lactobacillus* gradually colonized broiler intestines with increased broiler age. Moreover, the intestinal microbial community trended towards maturation over time and the fecal microbial communities were more complex than the jejunal and cecal communities.

To obtain optimal intestinal microbial communities for the ideal growth and health of broilers, the supplementation of feed additives to diets has become inevitable for regulating intestinal microbial communities. In this study, the relative abundances of specific microbial populations in Cobb broiler feces, jejuna, and ceca were affected by EOA1 and EOA2 dietary additives. For example, EOA1 and EOA2 treatment primarily affected the relative abundances of Lactobacillales (including *Enterococcus*, *Lactobacillus*, and *Aerococcus*) in the feces and jejuna of broilers, and significantly reduced

Proteobacteria abundances (including those of *Ralstonia pickettii* and Alcaligenaceae) after 28 days of age. Among these, *Enterococcus* were significantly enriched in feces at specific growth stages after addition of VM, EOA1, and EOA2 to diets, while *Aerococcus* were only significantly enriched in 14-day-old broilers after the addition of VM to diets. As the intestinal microbial communities matured, certain *Lactobacillus* strains were enriched in feces and jejuna after diet supplementation with VM, EM, EOA1, and EOA2. The genera *Enterococcus* and *Aerococcus* contain opportunistic pathogens that can co-exist with their hosts and are resistant to antibiotics [43, 44], and their presence may be consistent with the instability of microbial communities during the early developmental stages of broilers [35]. However, the flora that were enriched in broiler ceca after diet supplementation with VM, EM, EOA1, and EOA2 were mostly Clostridia (primarily including Lachnospiraceae, Oscillospiraceae, and Ruminococcaceae) and Bacteroidia (primarily including *Bacteroides*, *Alistipes*, *Rikenella*, and *Parabacteroides*). Clostridia and Bacteroidia are usually considered beneficial flora for intestines. Among the above taxa, Lachnospiraceae, Oscillospiraceae, and Ruminococcaceae can produce SCFAs through fermentation including butyric acid, acetic acid, and propionic acid, which then provide energy for the regeneration and repair of intestinal epithelial cells, inhibit the proliferation of pathogens, and promote intestinal health. [30, 45–47]. *Alistipes* and *Rikenella* belong to the Rikenellaceae family [48], and *Alistipes* are considered potential SCFA-producing bacteria [49]. whereas *Rikenella* can produce glucose and lactose [48]. *Bacteroides* and *Parabacteroides* abundances are also closely related to host immune system function [50, 51]. Chen et al. reported that both essential oil (including 78.3% cinnamic dehyde, 4% isophorone, and 2.7% eugenol) and VM treatment increased the relative abundances of *Bacteroides*, *Alistipes*, and other Bacteroidetes taxa in broiler ceca [52]. Further, the addition of citric acid and butyric acid to broiler feed has been suggested to create acidic intestinal environments that are conducive to the proliferation of *Lactobacillus*, in addition to the inhibition or killing of gram-negative bacteria such as *E. coli* and *Salmonella* [53, 54]. In summary, EOA1 and EOA2 dietary supplementation can effectively inhibit the proliferation of *Ralstonia pickettii* and Alcaligenaceae in Cobb broiler jejuna at specific growth stages, promote the colonization and growth of beneficial bacteria such as *Lactobacillus*, Clostridia, and Bacteroidia, and maintain intestinal microbiota balance.

Intestinal microbiota are involved in the metabolism and absorption of many nutrients and play key roles in maintaining the integrity of the intestinal barrier structure, immune regulation, and defenses against pathogen invasion [25, 55]. The reasons underlying these beneficial effects may rely on the activities of certain intestinal microbiota (e.g., *Lactobacillus*) that can ferment carbohydrates to produce vitamins, produce various enzymes, and produce SCFAs, in addition to other substances [56]. These substances can directly migrate to colon, liver, and muscle tissues to then participate in host circulatory systems, but can also be metabolized by host enzymes to produce signal molecules that regulate host immune system and energy metabolism [57]. For example, SCFAs can lower intestinal pH to create an acidic environment that regulates host physiological processes [58], while various enzymes released into the intestinal lumen have potentially synergistic effects on digestion and can facilitate intestinal absorption of nutrients [59]. In this study, functional inference analysis revealed that both EOA1 and EOA2 were inferred to enhance the prevalence of biological pathways related to metabolism, including lipid metabolism, enzyme

families, and energy metabolism, in 42-day-old broilers, while concomitantly attenuating functions related to cellular processes, genetic information processing, and environmental information processing. Butyric acid has been reported to improve the digestion and absorption processes of broilers, enhance body anabolism, and improve growth performance [53, 60]. Further, citric acid can improve protein digestion and absorption, reduce the production of growth-inhibiting microbial metabolites (e.g., ammonia), facilitate mineral absorption [54], and directly participate in body metabolism via the immediate synthesis of adenosine triphosphate (ATP) through the tricarboxylic acid cycle in response to adverse environmental conditions [61]. Pham *et al.* demonstrated that a mixture of encapsulated essential oils and organic acids could improve broiler growth by modulating intestinal microbial communities, enhancing intestinal barrier functions, modulating immune responses, and improving necrotizing enterocolitis-induced intestinal damage [13]. Therefore, we hypothesize that EOA1 and EOA2 can improve Cobb broiler growth by modulating the structure of their intestinal microflora and affecting multiple pathways related to nutrient metabolism and absorption.

# Conclusion

In conclusion, changes in the jejunal, cecal, and fecal microbial communities of Cobb broilers were systematically investigated across different growth stages in this study. *Lactobacillus* was the dominant bacterial taxa in the fecal and jejunal communities and it gradually came to dominate communities with increased broiler age. In addition, EOA1 and EOA2 dietary supplementation was able to reduce the relative abundances of certain Proteobacteria in Cobb broiler jejuna for a specific period of time, promote the colonization and growth of beneficial bacteria (e.g., *Lactobacillus*, Clostridia, and Bacteroidia) in the intestine, and influence nutrient absorption and broiler growth by regulating biological processes related to metabolism. These data demonstrate that EOA1 and EOA2 supplementation have potential health benefits for Cobb broilers and can be applied as alternatives to antibiotics to improve the growth performance of broilers during production.

# Abbreviations

EOA1: Thymol-citric acid; EOA2: Thymol-butyric acid; EM: Enramycin; VM: virginiamycin ; SCFAs: Short chain fatty acids; CTAB: Cetyltrimethyl ammonium bromide; PCoA: Principal coordinates analysis; LEfSe: Linear discriminant analysis effect size；LDA: Linear discriminant analysis

# Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and material

All data generated or analyzed during this study are available from the corresponding author by request.

### Competing interests

The authors declare no competing interests.


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### Author contributions

JQ, HL, XL, and ZS designed the experiments. ZS, KW, XL, and QW performed the experiments. ZS, XL, and HL analyzed the experimental data. JQ and HL wrote this paper.


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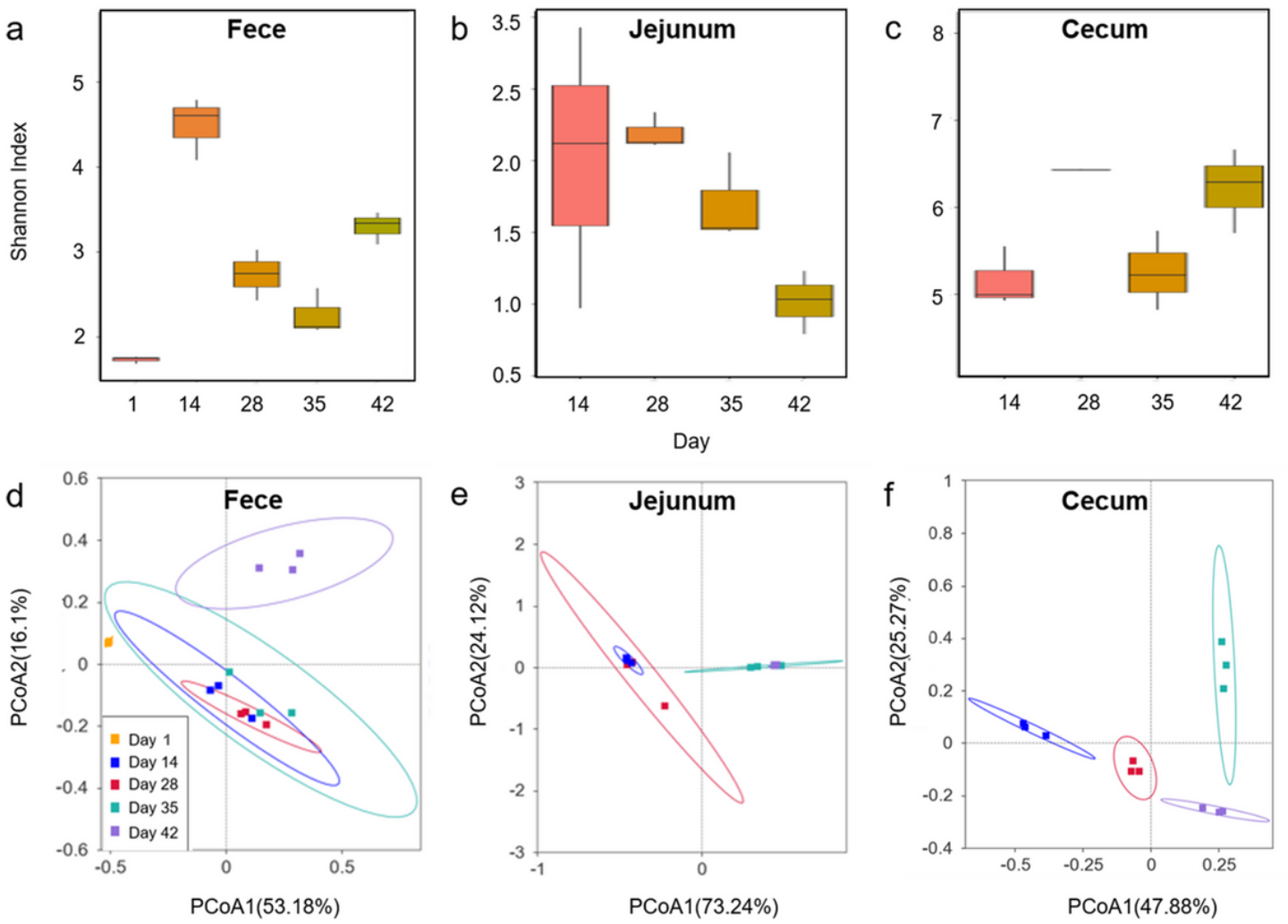
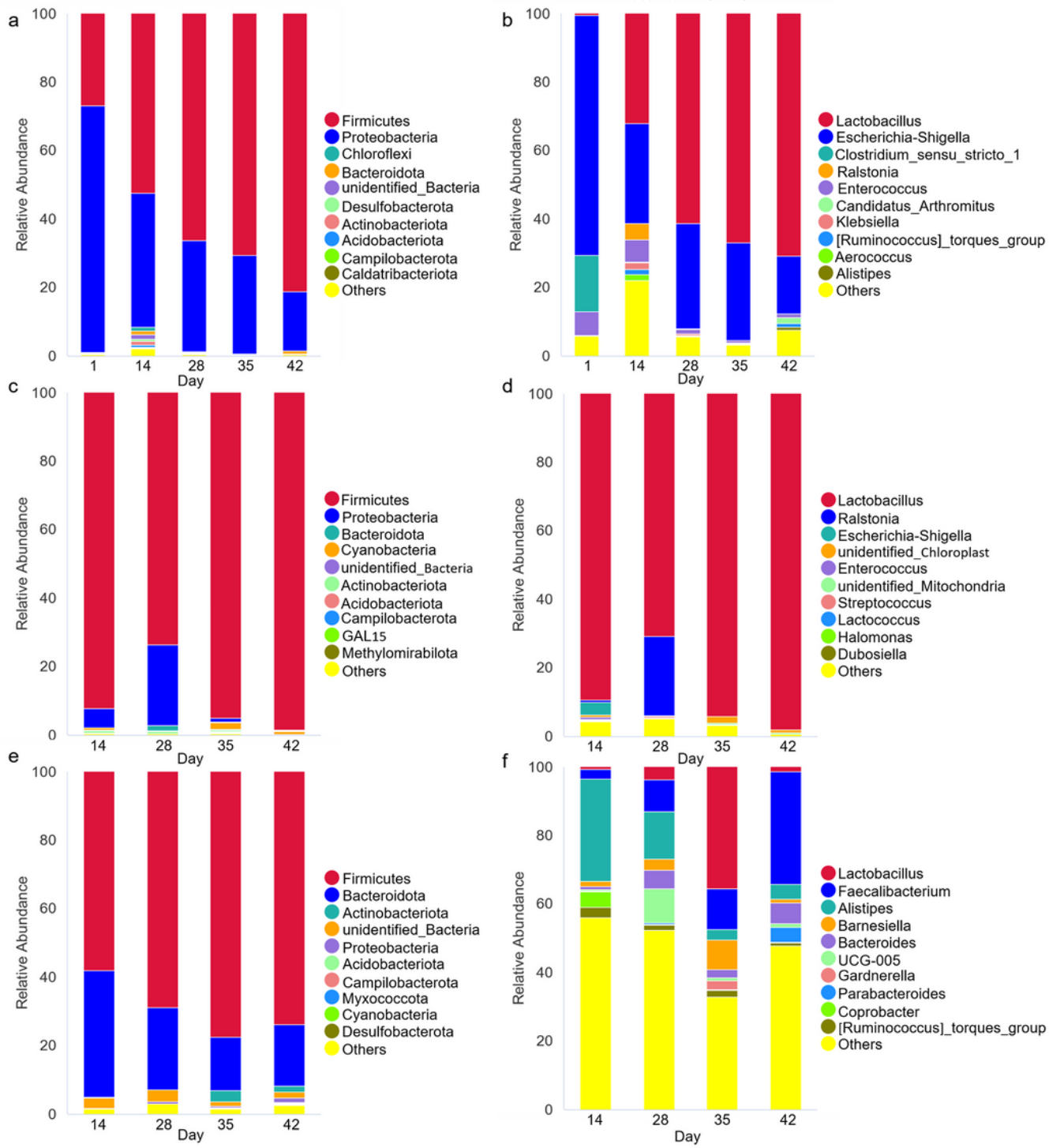
# Figures



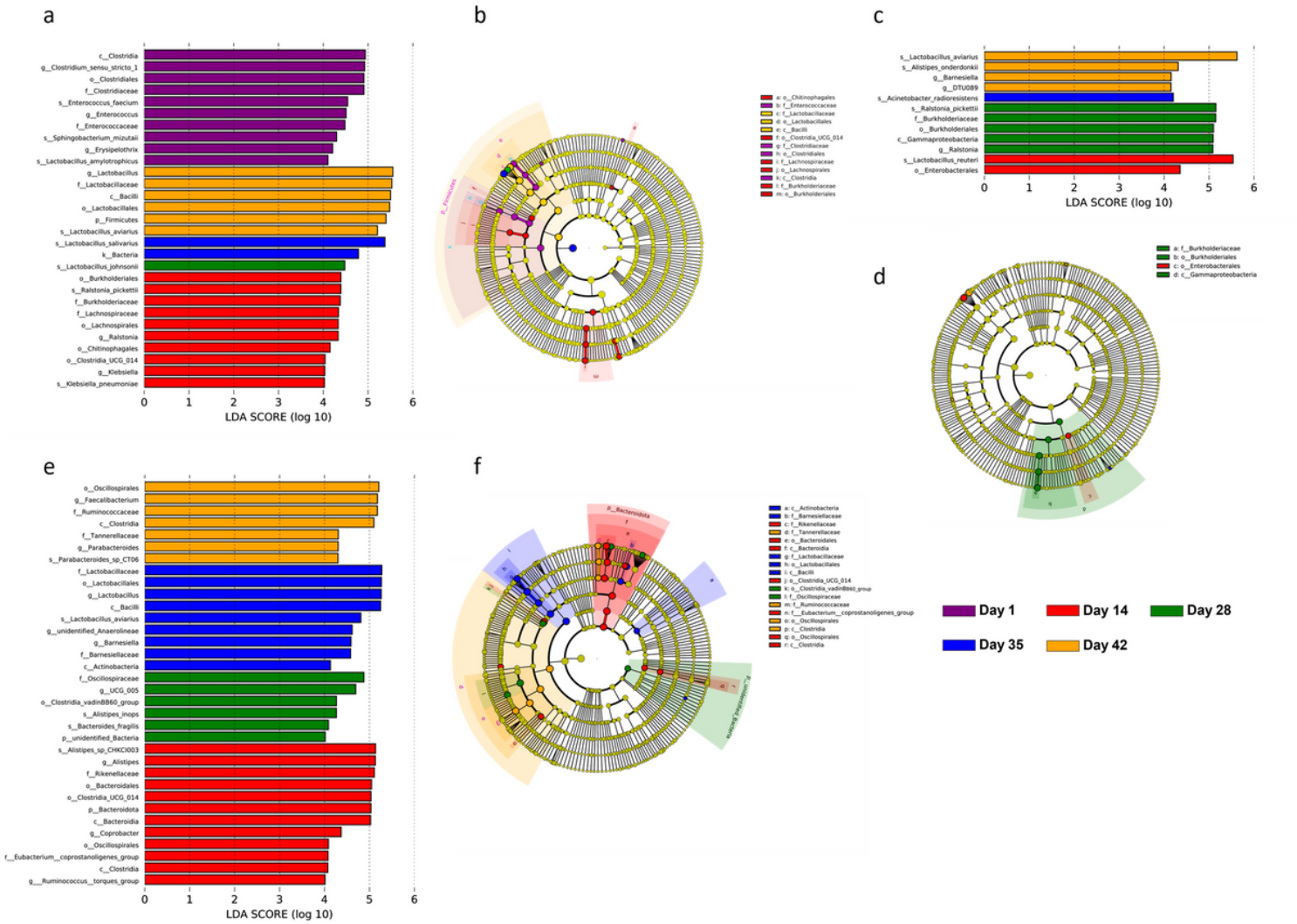

## Figure 1

Changes in the Cobb broiler fecal, jejunal and cecal microbial communities at different ages. (a-c) Longitudinal changes in the microbial community diversity (Shannon index) of Cobb broilers at different ages. (d-f) Longitudinal changes in the microbial structures of Cobb broilers at different ages.



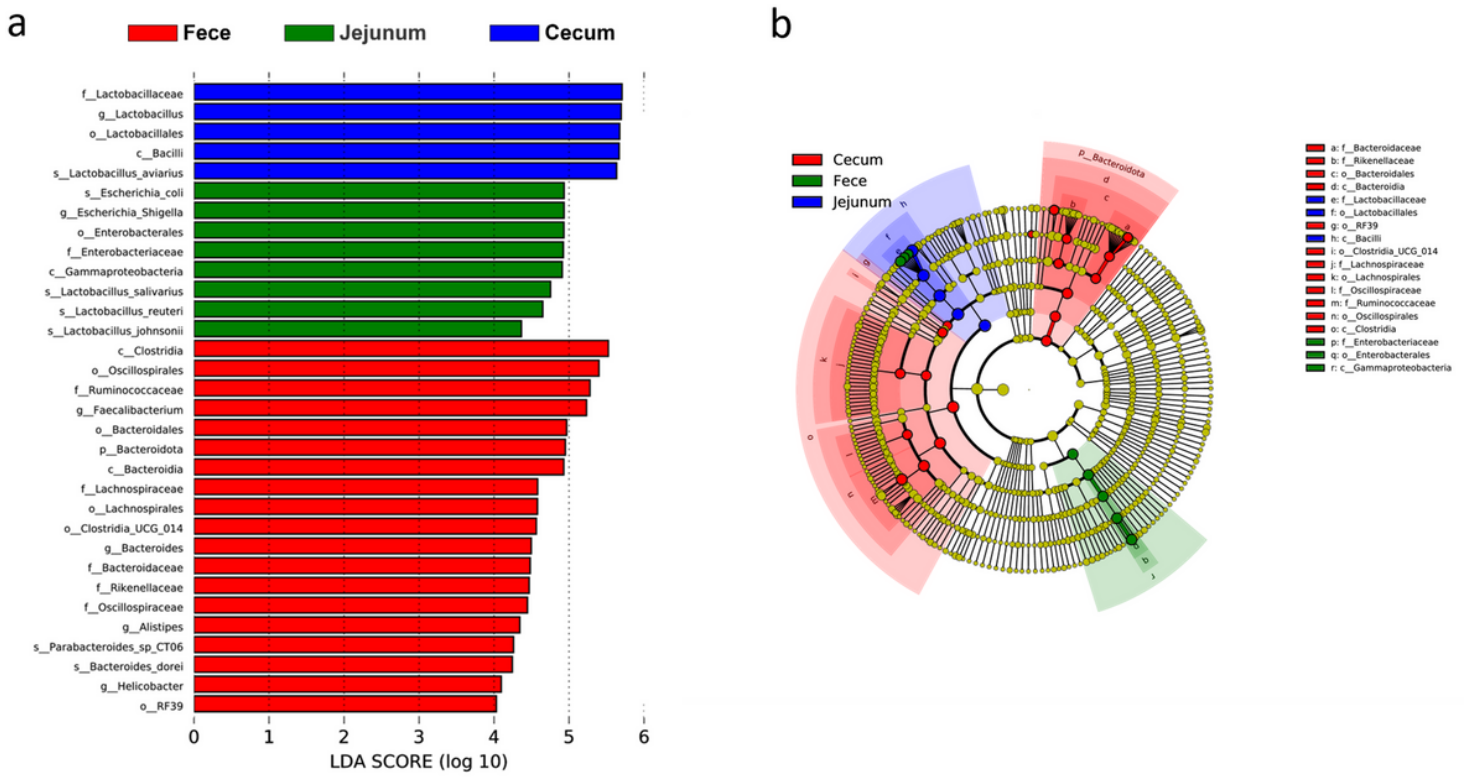

## Figure 2

The microbial species composition of Cobb broiler feces, jejuna and ceca at different ages. (a, c, e) Histogram of species relative abundance at the phylum level. (b, d, f) Histogram of relative species abundance at the genus level.



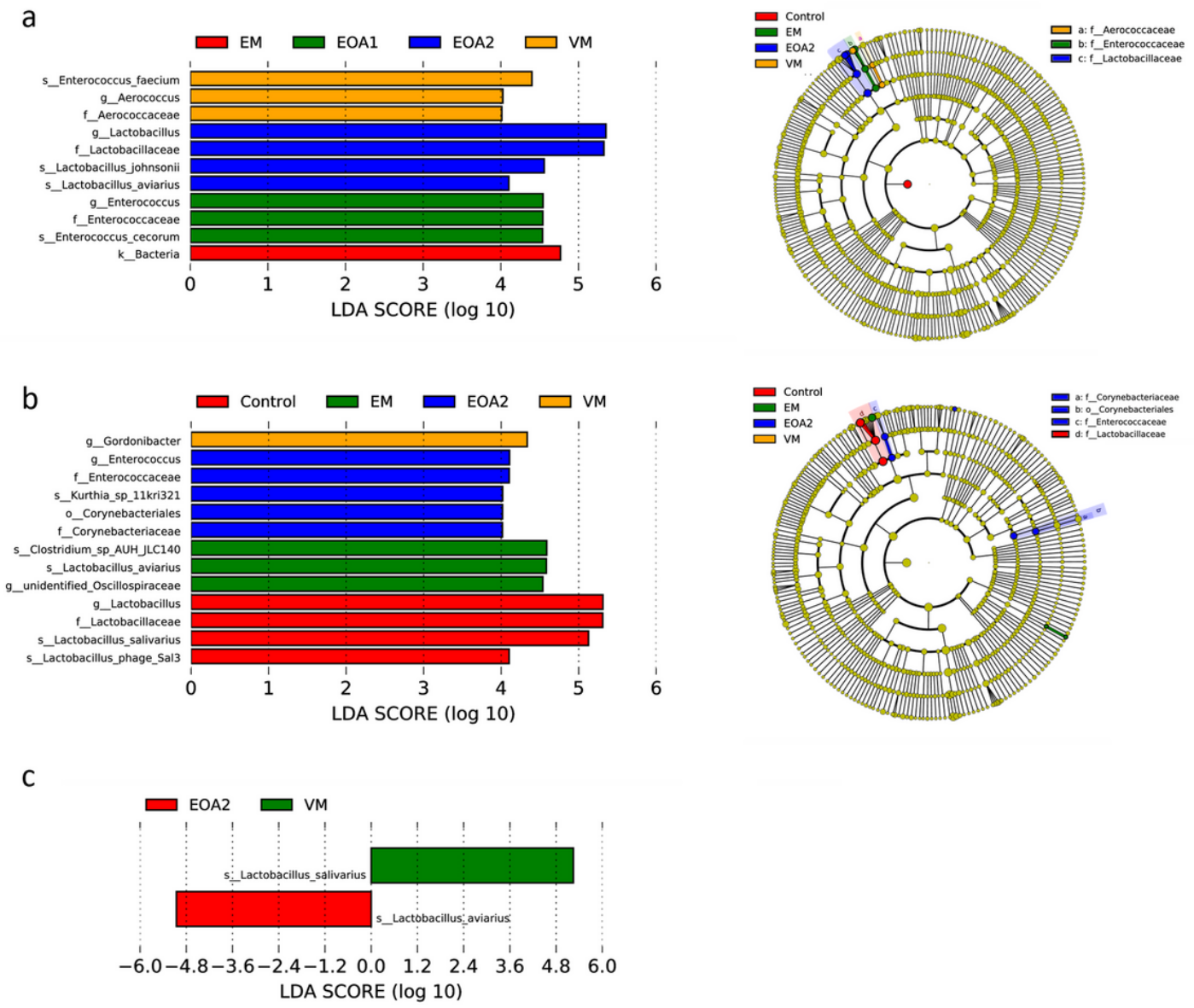

## Figure 3

Differences in the Cobb broiler fecal, jejunal and cecal microbial species composition at different ages. (a, b) LEfSe analysis for the microbial communities of Cobb broiler feces at the phylum to species taxonomic levels among day 0, 14, 28, 35 and 42. (c, d) LEfSe analysis for the microbial communities of Cobb broiler jejuna at the phylum to species taxonomic levels among day 14, 28, 35 and 42. (e, f) LEfSe analysis for the microbial communities of Cobb broiler ceca at the phylum to species taxonomic levels among day 14, 28, 35 and 42.



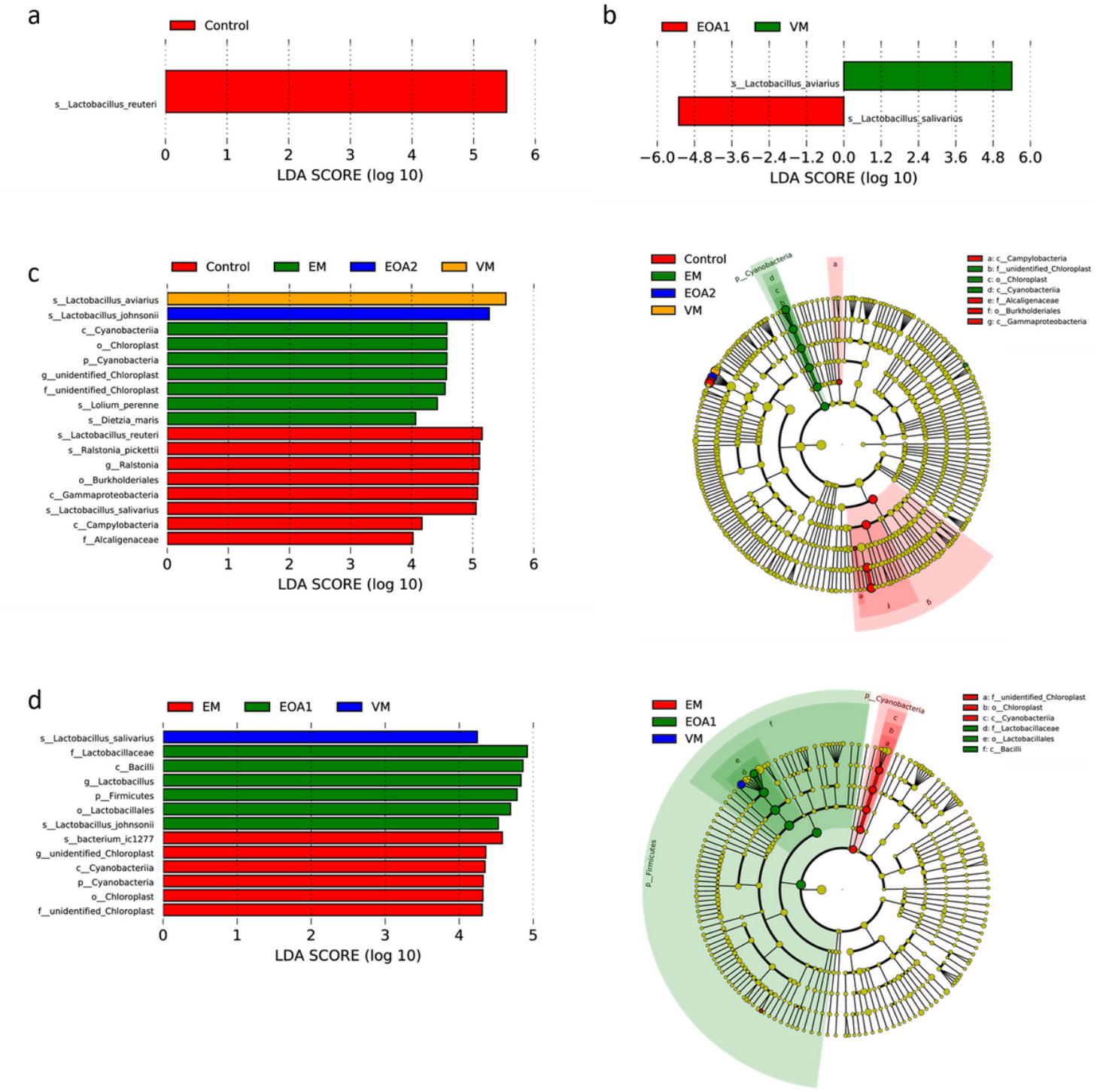

## Figure 4

Differences of microbial communities among different intestinal sites (jejuna, ceca, and feces) of 42-day-old broilers. (a, b) LEfSe analysis of microbial communities at the phylum to species levels among feces, jejuna and ceca on day 42.



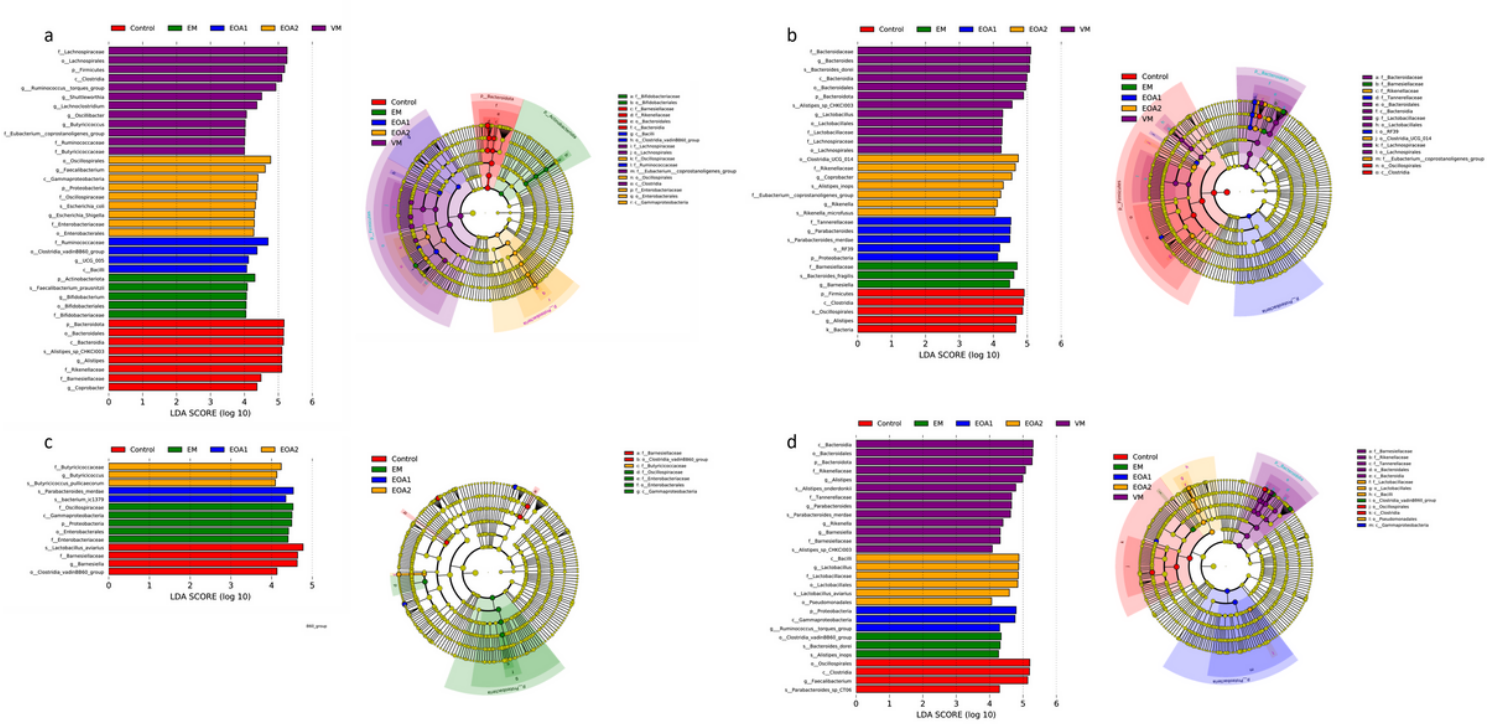

## Figure 5

Differences in fecal microbial changes after the diet supplementation with VM, EM, EOA1 and EOA2 at different ages. LEfSe analysis of fecal microbial communities from phylum to species levels among VM, EM, EOA1 and EOA2 groups on day 14 (a, b), 28 (c, d) and 35 (e).




## Figure 6

Differences in jejunal microbial changes after the diet supplementation with VM, EM, EOA1 and EOA2 at different ages. LEfSe analysis of jejunal microbial communities at the phylum to species levels among VM, EM, EOA1 and EOA2 groups on day 14 (a), 28 (c), 35 (b) and 42 (d).




## Figure 7

Differences in cecal microbial changes after the diet supplementation with VM, EM, EOA1 and EOA2 at different ages. LEfSe analysis of cecal microbial communities from phylum to species levels among VM, EM, EOA1 and EOA2 groups on day 14 (a), 28 (b), 35 (c) and 42 (d).

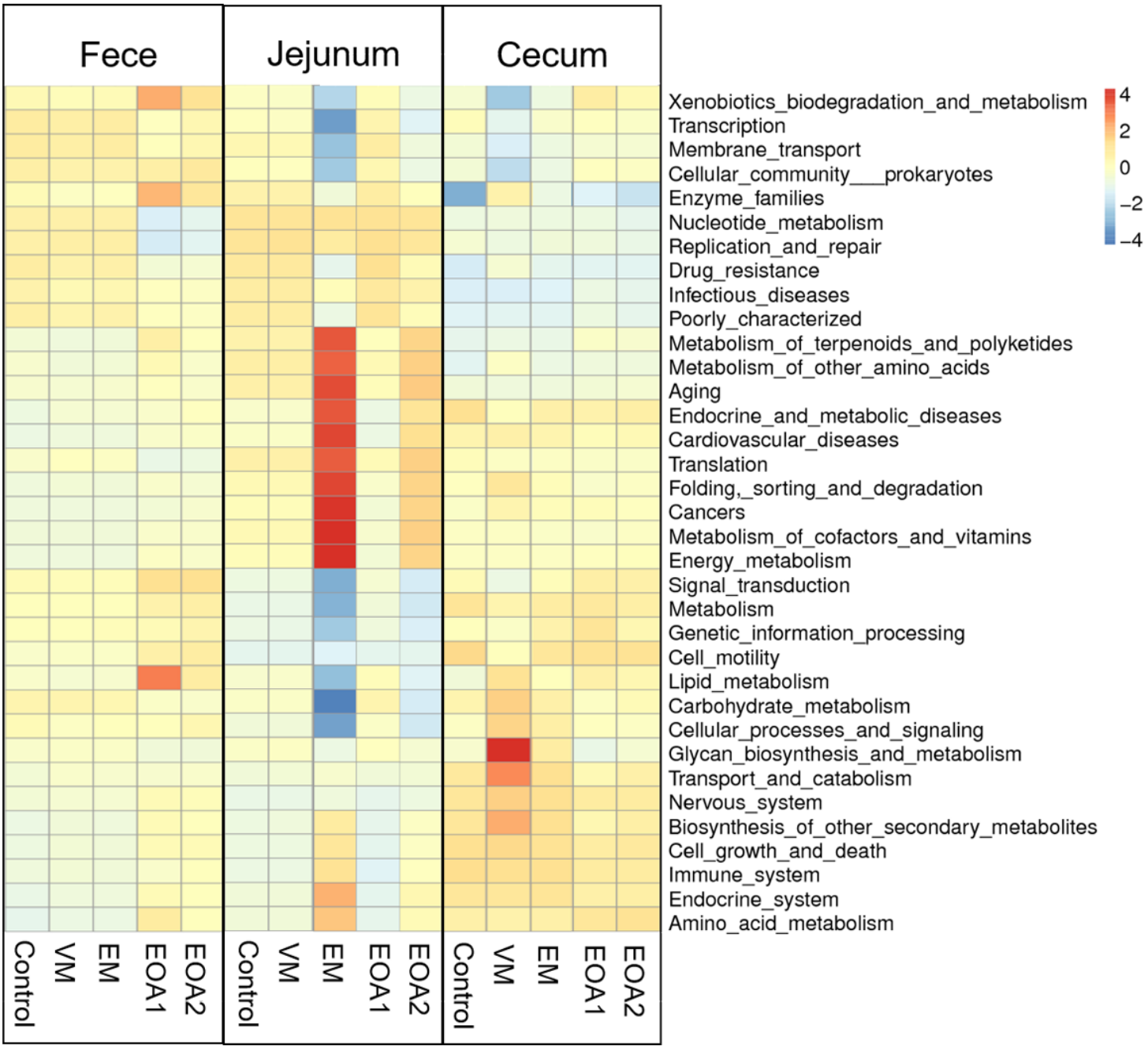



**Figure 8**

Effect of different feed additives on microbial functions of Cobb broiler feces, jejuna and ceca.

# Supplementary Files

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- additionalmaterials.docx