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RESEARCH ARTICLE

Effect of Flavonoids from *Eupatorium odoratum* L. on Immunoglobulins and Cecal Microflora in Broilers

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ABSTRACT

The effects of total flavonoids from *Eupatorium odoratum* L. (TFEO) on serum (IgM, IgG) and duodenal mucosa (Secretory IgA–SIgA) immunoglobulins and cecal microflora composition in broiler chicken were investigated. Test groups received diets supplemented with 100, 200, or 400 mg/kg TFEO for 21 and 42d. At 21d, IgM levels were higher in the 200 and 400 mg/kg groups compared to control group. IgG and SIgA levels were increased in all test groups ($P<0.05$). At 42d, IgG content in all test groups were higher ($P<0.05$), IgM in all test groups was higher ($P>0.05$). SIgA in all test groups increased at 200 and 400 mg/kg ($P<0.01$) >100 mg/kg ($P<0.05$) compared to the controls. Significant increases ($P<0.05$) in *Firmicutes* phylum and *Bifidobacterium*, *Lactobacillus* and *Lachnospira* genera gut microbes and significant decreases in pathogenic *Escherichia* and *Clostridium* of the test groups were observed at 21 and/or 42d. TFEO flavonoids are suggested for use as a feed additive for broilers to improve cecal microflora and humoral immunity.

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INTRODUCTION

Eupatorium odoratum L., Asteraceae, commonly called "Siam weed", is native to the Andes Mountains. It was officially recorded in China in southern Yunnan Province in 1934 (Omokhua *et al*., 2016), and was listed as an invasive species in 2003 (Ge, 2003). *E. odoratum* has been used as a Chinese medicinal herb for over 2000 years and many active components have been identified, such as flavonoids, alkaloids, terpene, phenols, quinonoids and phytosterols (Omokhua *et al*., 2016). The total flavonoids from *E. odoratum* (TFEO) have been identified as kaempferol-3-methoxy (I), rhamnetin (II), tamarixetin (III), quercetin (IV), kaempferol (V), apigenin (VI), luteolin (VII) and dihydrokaempferide (VIII) (Yuan *et al*., 2007). Pharmacologically, TFEO have a wide range of biological properties including anti-inflammatory, antibacterial, antioxidant, anticonvulsant, and cardiovascular and neurological functions (Yuan, 2007).

The intestinal microbial flora of broilers plays an important role in growth performance and health of birds (Porras, 2015). It is generally assumed that flavonoids are poorly absorbed from the gastrointestinal tract in its native form and must be hydrolyzed by intestinal microflora to its aglycones (Hollman, 2004). Serum immunoglobulins (IgM, IgG) are the main immunoglobulins in broiler serum. Secretory IgA (SIgA) produced by B cells is the main immunoglobulins in broiler gut and secreted to the surface of the intestinal mucosa (Song, 1983). Additives are widely used in animal production to improve feed quality, promote animal growth, break down anti-nutritional factors, absorb toxins and alleviate nutritional deficiencies (Mendel *et al*., 2017). However, the effect of TFEO on animals has not been reported. This study investigated the effects of TFEO on humoral immunity [IgM and IgG in serum and secretory immunoglobulin A (SIgA) in duodenal mucosa], and cecal microflora in broilers.

§These authors contributed equally to this work.

MATERIALS AND METHODS

Sample collection, preparation and flavonoids extraction: *E. odoratum* was dried at room temperature, made into a powder, packed in vacuum polyethylene bags and stored at 4ºC. TFEO was extracted and quantified as described by Zheng *et al*. (2015) and stored at 4ºC until use.

Broiler grouping and diet: One-day-old Arbor Acre chickens (n=96) were allotted to a control and 3 treatment groups of 24 birds each (equal numbers of males and females). Broilers in Group 1 (control) were fed with a basal diet, and for the experimental groups 2, 3 and 4, 100, 200 and 400 mg/kg TFEO were added, respectively. Samples from the broilers were collected on days 21 (stage A) and 42 (stage B).

Immunoglobulin analysis – Serum IgM and IgG and duodenal Secretory IgA: On days 21 and 42, respectively, blood samples were collected via the brachial vein of 8 broiler chickens per group, centrifuged at 2×1000*g* for 15 min at 4°C and serum was stored at −20ºC (Daneshmand *et al*., 2017). Serum IgM and IgG were determined using an ELISA kit (Jimian Biotechnology Co., Shanghai, China).

The SIgA level was determined according to the method of Zhu *et al*. (2017). Briefly, the duodenum was collected and washed three times with ice-cold phosphate-buffered saline (PBS) to remove mucus and digesta. The duodenal mucosa was gently scraped with a glass slide, snap-frozen in liquid nitrogen, and stored at −80°C until analyses. The SIgA was determined using an ELISA kit (Jimian Biotechnology Co.).

Cecal microflora total DNA preparation: Cecal contents were collected from 4 broiler chickens per group. The cecal contents were washed, centrifuged with PBS, and stored at −80°C till further analysis. Total DNA of cecal microflora was extracted by E.Z.N.A Soil DNA Kit (OMEGA, USA) according to the manufacturer's instructions. DNA was stored at −20 ºC until use.

PCR amplification and Miseq sequencing: For total DNA, universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used (Dennis *et al*., 2013), The PCR conditions were: initial denaturation at 95°C for 3 min 30 s, annealing at 55°C for 30 s, followed by 27-cycle extension at 72°C for 45 s, with a final extension phase at 72°C for 10 min. PCR product was assessed by 2% agarose gel electrophoresis and recycled by an AxyPrep DNA gel Recovery Kit (AXYGEN, USA). The end products were sequenced through Miseq PE300 at the Shanghai Major Biopharmaceutical Technology Co. (http://www. majorbio. com).

Bioinformatics: This was carried out in 3 steps. Step 1: Miseq sequencing data were subjected to 2-terminal sequencing according to the overlap relationship between paired-end reads, and pairing reads were spliced and merged into a sequence and quality control filtration conducted. Valid sequences were obtained according to the barcode of sequence from beginning to end and sequence of the primer and then the sequence direction was corrected. Step 2: The non-repetitive sequence from optimized sequence of step 1 was extracted, a single sequence removed, and clustering performed to <97% similarity to obtain Operational Taxonomic Unit sequences. Step 3: Taxonomy was conducted using a Bayesian-algorithm, and the microbial community composition of samples was determined at phylum and genus levels (Cole *et al*., 2009).

Statistical analysis: Immunoglobulin content data were analyzed using SPSS Statistics v. 19.0 (SPSS Inc., Chicago, IL, USA). Values are expressed as means ± S.E. The data on cecal microflora composition were based on individual broilers. Rarefaction analysis of Operational Taxonomic Units was conducted using Mothur, version v.1.30.1 (Schloss *et al*., 2011). Ribosomal Database Project (RDP), one of the most widely used tools to classify 16S rRNA sequences. RDP classifier Bayes algorithm was used in cluster analysis and the Bray−Curtis distance algorithm was used to measure community differences. Statistical significance was set at $P<0.05$.

Ethical considerations: All broilers were housed in as 4 groups in an environmentally-controlled environment. The broilers were given free access to feed and water during the 42-day experiment. The broiler research carried out in this study was approved by the Animal Ethics Committee of Guangdong Ocean University (Approval No: NXY2015003). The Animal Research: Reporting of In Vivo Experiment (ARRIVE) guidelines were adhered to.

RESULTS

Effects of TFEO on immunoglobulin: At day 21, IgM concentration was increased significantly in the 200 and 400 mg/kg groups compared with the control group, but this was not evident at 42 days (Table 1). At day 21, IgG concentration increased significantly in all experimental groups, and at day 42, a significant ($P<0.05$) increase was also observed in all test groups. SIgA was also increased significantly ($P<0.05$) in test groups at day 21 but at 42 days increases were particularly significant for the 200 and 400 mg/kg groups ($P<0.01$) >100 mg/kg group ($P<0.05$) compared with the control (Table 1).

Table 1: Immunoglobulin concentrations (µg/mL) in broilers fed total flavonoids from *Eupatorium odoratum* L. (TFEO) for 21 (stage A) and 42 days (stage B). IgM and IgG refer to the respective serum immunoglobulin, and SIgA to secretory immunoglobulin in cecal mucosa. '*' P<0.05, '**' (P<0.01). Group 1 refers to the control group (fed a corn–soybean basal diet), and Groups 2, 3 and 4 are test groups fed basal diets supplemented with 100, 200, and 400 mg/kg TFEO, respectively

Stage	Immuno-globulin	Group 1	Group 2	Group 3	Group 4
A	IgM	236.50±10.34	248.67±9.87	276.67±9.45*	281.34±10.52*
	IgG	254.75±10.37	334.92±9.49*	344.42±11.82*	369.25±9.53*
	SIgA	14.76±0.35	18.35±0.40*	19.23±0.39*	20.98±0.42*
B	IgM	162.38±11.34	165.11±10.98	172.37±11.23	179.36±9.53
	IgG	462.36±10.97	494.54±9.37*	519.36±10.75*	530.22±10.49*
	SIgA	21.83±0.82	25.36±0.87*	30.54±0.53**	35.87±0.66**

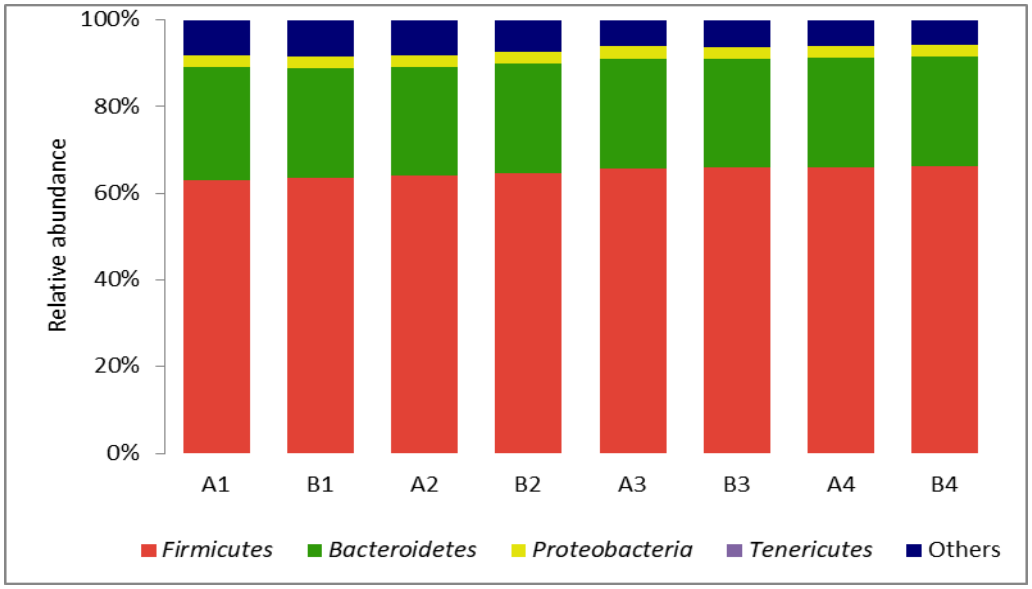

Fig. 1: Effect of total flavonoids of *Eupatorium odoratum* L. (TFEO) on microbial phyla composition in the cecum of broiler chickens on days 21 (stage A) and 42 (stage B). A1 and B1 (controls); A2 and B2 (100 mg/kg), A3 and B3 (200 mg/kg), A4 and B4 (400 mg/kg) are test groups supplemented with TFEO.

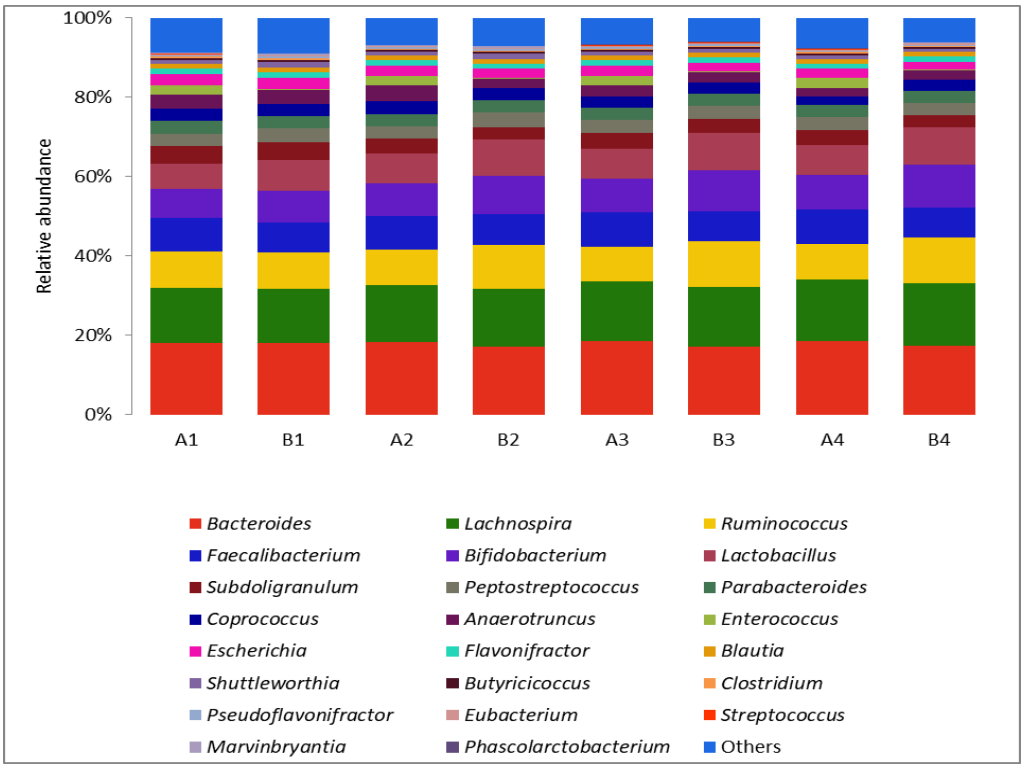

Fig. 2: Effect of total flavonoids of *Eupatorium odoratum* L. (TFEO) on microbial genera composition in the cecum of broiler chickens on days 21 (stage A) and 42 (stage B). A1 and B1 (controls); A2 and B2 (100 mg/kg), A3 and B3 (200 mg/kg) and A4 and B4 (400 mg/kg) are test groups supplemented with TFEO.

Effects of TFEO on cecal microflora: The *Firmicutes>Bacteroidetes>Proteobacteria* phyla were dominant in the broiler cecal microbial community (Fig. 1). At 21 and 42 days, the *Firmicutes* of the test groups increased compared with the controls ($P>0.05$).

At days 21 and 42 (Fig. 2), *Bifidobacterium* and *Lactobacillus* in test groups increased significantly ($P<0.05$) compared with the control group. At day 42, *Lachnospira* in test groups increased while *Escherichia* and *Clostridium* decreased significantly ($P<0.05$) compared with the control group (Fig. 2).

DISCUSSION

TFEO and immunoglobulins: Humoral immunity involves antibody production and associated immune responses, and immunoglobulin content has been used widely to evaluate it (Kong *et al*., 2004). As a part of the adaptive humoral immune system, plasma B cells produce large quantities of SIgA, which is the most important immunoglobulin in external secretions (Gutjahr, *et al*., 2016). Following secretion of SIgA onto the surface of intestinal mucosa, a protective membrane is formed,

which can agglutinate and capture the antigen on the mucosal surface, and effectively resist the invasion of intestinal antigens (Brandtzaeg, 2009).The main functions of SIgA are inhibition of polymer absorption and inflammatory response of other immunoglobulins and the binding of allergens to mucosal target cells (Zhao *et al.*, 2016). It has been reported that 7-day-old chickens have few SIgA cells and that cell numbers gradually increase over time and peak at around 49-50 days (Lammers *et al.*, 2010). The SIgA numbers in test groups were significantly elevated compared with the control group at both 21 and 42 days. It is presumed that the increase in SIgA on exposure to TFEO improves broiler intestinal health. However, it is not known how the flavonoids affect SIgA.

Immunoglobulin M (IgM), the largest pentameric immunoglobulin, is used as a marker of recent primary infection and IgM provides a crucial first line of defense for the immune system, and its ability to agglutinate pathogens enhances phagocytosis and clearance (Padmalatha and Ronald., 1999). IgG is the main immunoglobulin in poultry serum and the concentration in adult poultry serum varies from 4.1 to 7.3 mg/mL (Warner *et al*., 1969). Effects of flavonoids from different sources on IgM and IgG have been reported. Fan *et al*. (2014) showed that propolis flavone microemulsion (flavone is one of the most important pharmacologically active constituents in propolis and is thought to be responsible for many of its biological and pharmacological activities) significantly promoted splenocyte proliferation in the mouse and secretion of IL-2 and IFN-γ *in vitro*, while at high and medium doses *in vivo*, it significantly increased the thymic and splenic indices, enhanced splenocyte activity and improved the serum IgG and IgM content. Liu *et al*. (2013) found that mice exposed to high doses of total flavonoids from leaves of *Choerospondias axillaris* exhibited enhanced immune function, with improvement in phagocytic function of the mononuclear macrophage, cellular immunity, and humoral immunity. Our results agree with all of the above literature in that flavonoids increase the content of immunoglobulins in animals. Our results in general showed that the IgM in broiler groups given mid and high-doses of TFEO increased significantly compared with the control group. Overall, the results showed positive effects of TFEO on immunoglobulin synthesis, which is similar with Fan *et al*. (2014) and Liu *et al*. (2013) research. However, the content of IgM decreased at 42 days compared with that at 21 days, which is puzzling; but the IgG concentration was significantly higher than the control group at 42 days. We believe that this may due to an adaptation by the immune system and the dominance of IgG beyond early stages of life. IgM is one of the dominant antibodies produced in early life and reaches a peak at around 4–8 days and declines thereafter (Song, 1983). IgM increased indicating a positive effect of TFEO at 21d > 42d.

TFEO and intestinal microflora: The gut microflora play an important role in the absorption, distribution, metabolism, and excretion of flavonoids have the ability to modify the gut microbial balance acting in a prebiotic capacity (Porras, 2015). The microflora of broilers was mostly from the *Firmicutes* and *Bacteroidetes* phyla which is in agreement with the findings of Qin *et al.* (2018). According to Braune (2016), the *Firmicutes* phylum contains many genera including *Lactobacillus*, *Enterococcus* and *Lachnospira* all of which play a role in the metabolism of dietary flavonoids. In our experiment, *Firmicutes* increased significantly in broilers supplemented with 100, 200, 400 mg/kg TFEO for 42days which may be related to an increase in *Lactobacillus* and *Lachnospira*. However, this was not apparent at 21 days possibly because the gut microflora in the intestine and cecum takes at least 4–6 weeks to respond and stabilize following dietary changes (Daniele, 2012).

Our results showed that *Lactobacilli* and *Bifidobacteria* increased significantly at 21 and 42 days. *Lactobacilli* and *Bifidobacteria* are beneficial bacteria in the broiler cecum that can improve the health and growth of broilers and are increased on supplementation of the diet with probiotics such as *L. acidophilus* LAP5, *L. casei* L21, *L. fermentum* P2 and *P. acidilactici* LS) (Yu, 2015). Hence TFEO may improve the composition of gut microflora by increasing probiotics. Moreover, Amaretti *et al*. (2015) observed that *Lactobacilli* and *Bifidobacteria* can also metabolize flavonoids. Yang *et al.* (2014) found that black berry juice rich in flavonoids mostly contained anthocyanins, proanthocyanidins, all of which promoted the growth of *Lactobacilli* strains confirming the capability of the flavonoids to increase *Lactobacilli* and improve the intestinal microflora community. Serra *et al*. (2006) showed that the tea flavonoids contained epicatechin, catechin, 3-O-methyl gallic acid which can suppress the growth of pathogenic bacteria including *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides* spp. in humans. *Clostridium perfringens* is the main etiological agent of necrotic enteritis in broilers (Li *et al*., 2018). The *Clostridia* decreased in broilers given feed supplemented with TFEO at 100, 200 and 400 mg/kg compared with the control group at 21 days, and significantly decreased at 42 days, which shows that TFEO can inhibit the *Clostridia* in broiler cecum, and thereby prevent diseases such as necrotic enteritis.

Escherichia decreased in test groups supplemented with 100, 200 and 400 mg/kg TFEO compared with the control group on day 21 and significantly decreased at 42 days. *Escherichia* is a genus of Gram-negative, non-spore-forming, facultative anaerobic, rod-shaped bacteria belonging to the family *Enterobacteriaceae*. They are common inhabitants of the gastrointestinal tract of warm-blooded animals. However, a number of species of *Escherichia* are pathogenic (Hogan, 2010). Lee *et al*. (2010) noted that flavonoids (quercetin and naringenin) can inhibit the growth of *E. coli*. Our study has shown that TFEO may be acting as an antimicrobial feed additive that promotes the growth of beneficial probiotics and inhibit harmful bacteria in the gut, thereby improving the gut microflora community and promoting health effects.

Lastly, the immunoglobulins, particular SIgA increased along with beneficial bacteria increased while the pathogenic bacteria decreased. Thus, we believe that TFEO in the broiler gut influences the growth of microflora, promotes especially the growth of beneficial bacteria and suppress the pathogenic bacteria. Associated with this is that TFEO influences SIgA of intestinal mucosal surface which protects the internal environment

by suppressing the growth of pathogenic bacteria, the mechanism of which has still not been clearly established.

Conclusions: Supplementing the Arbor Acre broiler diet with TFEO increased the serum IgM and IgG and the duodenal SIgA. TFEO improved the composition of the intestinal microflora community in broilers and hence could be used as an herb-resourced feed additive for broilers.

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Authors contribution: Q-HZ, F-HN, J-JC and RG designed the study and interpreted the results and revised the paper. Q-HZ, F-HN and X-NW carried out the experiments and wrote the original manuscript. F-HN and X-NW also took part in the experiment and collected the test data. H-YL and H-CRW performed the data analyses. Q-HZ, J-JC and RG interpreted the results and edited the manuscript.

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