Effect of plant extracts derived from thyme and chamomile on the growth performance, gut morphology and immune system of broilers fed aflatoxin B1 and ochratoxin A contaminated diets

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ABSTRACT
This study was conducted to evaluate the ability of chamomile flower extract (CFE) and thyme-oil extract (TOE) for the preventing effect of ochratoxin A (OTA) and aflatoxin B1 (AFB) in broiler productivity, biochemical parameters, gut morphology and immune response of broilers. The experiment was conducted as a 3 x 4 factorial arrangement, being the factors: phytogenic additives (control, 3 g/kg diet of CFE, or 3 g/kg diet of TOE) and dietary contaminated diets (none, 0.50 mg/kg diet of AFB, 0.25 mg/kg diet of OTA, or 0.50 mg/kg diet of AFB + 0.25 mg/kg diet of OTA). Each of the 12 dietary treatments was fed to six replicate pens (10 birds/pen) from 0 to 28 d of age. Inclusion of phytogenic additives increased average daily gain (ADG) and average daily feed intake (ADFI), and decreased feed conversion ratio (FCR) from day 11 to 28 and day 0 to 28 in the birds fed OTA-AFB toxin diets. Significantly increased levels of HDL, IgG, IgM and anti-Newcastle disease virus (NDV) antibody titre were observed in the two essential oil-treated groups when compared with the control group. When compared with the control, relative weights of gizzard, liver and pancreas were significantly higher for broilers fed diets containing OTA alone. The results indicated that OTA and AFB toxins, alone or in combination, reduced villous height (VH) and ratio of VH to crypt depth in the jejunum. The levels of triglycerides, total cholesterol, HDL and plasma immunoglobulin, as well as NDV antibody titre were significantly decreased by AFB-OTA toxin combination. In summary, the combination of OTA and AFB impaired chick immune function even at combined concentrations as low as 0.25 mg/kg diet of OTA and 0.50 mg/kg diet of AFB. The inclusion of TOE and CFE can be an alternative to ameliorate the adverse effects of low doses of AFB and OTA in broiler diets.

HIGHLIGHTS
• Ochratoxin A and aflatoxin B1, alone or in combination, affect negatively productive performance and immune function.
• Dietary inclusion of chamomile flower extract (CFE) and thyme-oil extract (TOE) had positive effects on growth performance and immune response.
• Addition of CFE and TOE to diets can ameliorate the adverse effects of mycotoxins in broiler diets.

Introduction

Mycotoxins are bioactive secondary metabolites produced by fungi that contaminate grains in the field or during storage. Aflatoxin B1 (AFB), ochratoxin A (OTA) and T-2 toxin are some of the mycotoxins that can significantly compromise bird performance, increase susceptibility to infectious and parasitic diseases, and cause serious problems of reproductive leading to economic losses in the poultry industry. Mycotoxins produce a variety of diseases, collectively called ‘mycotoxicoses’, directly or in combination with other primary stressors such as pathogens.

Ochratoxin A, a nephrotoxic mycotoxin mainly produced by Aspergillus ochraceus and Penicillium viridicatum, has been found to occur in various grains, cereals and other plant products, animal feeds, meats and human tissues in countries throughout the world (Qu et al. 2017). Ochratoxin is more of a nephrotoxin than a hepatotoxic, and is a potent and severe nephrotoxic in broilers. It has teratogenic, mutagenic and
immunotoxic effects and is subjected to intestinal secretion and reabsorption via enterohepatic recycling (Bhatti et al. 2016). This mycotoxin causes reduction in growth, reduced feed intake and poor body weight gain, which finally bring about economic losses. Ochratoxin A causes congested and swollen kidneys, an increase in the size of liver, and reduction in the size of lymphatic organs such as spleen and bursa of Fabricius (Qu et al. 2017). OTA also causes severe immunosuppression which can be measured by antibody response to sheep red blood cell (SRBC) and the carbon clearance assay (Garcia et al. 2003). A particular danger is the presence of OTA residues in tissues of animals that have been given contaminated diets and which enter the human food chain. Consumption of meat and meat products from these animals is a major threat to human health (Heussner and Bingle 2015). When AFB and OTA are co-contaminants of poultry feed, they interact in a synergistic manner (Abidin et al. 2011). During dual exposure of these toxins, OTA prevents the major effects of AFB (i.e. fatty, yellow, enlarged and friable liver). This reduces the ability to diagnose aflatoxicosis in the field and the target organ in this interaction appears to be the kidney.

The most well-known approach for detoxification of mycotoxins involves the use of nutritionally inert adsorbents with the capacity to bind and immobilise mycotoxins in the gastrointestinal tract of animals, thus reducing their bioavailability (Campagnollo et al. 2015). Although this approach successfully eliminates the risk of certain mycotoxins such as the AFB, it does not work comprehensively on all of the mycotoxins relevant to the poultry industry. Biotransformation has been one of the proven approaches for the detoxification of the non-adsorbable mycotoxins by altering their molecular structure into non-toxic metabolites which are excreted (Ji et al. 2016). Therefore, suppression of mycotoxicoses requires an integrated approach from detection to detoxification. Several strategies (e.g. physical, chemical or biological) for detoxification and inactivation of toxicin are proposed to minimise harmful effects in animals and its consequences in order to prevent aflatoxicosis in human, livestock and poultry (Abidin et al. 2011). Recently, there is more interest to natural active biological compounds that derive from plants (Esper et al. 2014; Manafi et al. 2014; El-Khoury et al. 2017). It has been well documented that some secondary metabolites of herbs are effective in prevention of fungal growth because phenolic oil has anti-fungal and anti-bacterial properties (Manafi et al. 2014).

The aim of this study was to evaluate the effect of chamomile flower extract (CFE) and thyme-oil extract (TOE) in broiler diets contaminated with AFB and OTA (alone or in combination) on the performance, immune response and gut morphology of growing broilers.

Materials and methods

All procedures carried out in this experiment were reviewed and approved by the Animal Care and Use Committee of the University of Birjand.

OTA and AFB production

The fungi used in this study were prepared from the microbial treasures of Scientific and Industrial Research Organisation of Iran.

The required AFB1 was produced by contaminating rices with Aspergillus flavus (NRRL 2999). For this purpose, 1 mL of Aspergillus flavus suspension containing 7 × 10^6 fungal spores was added to rice and was cultivated for seven days at 28 °C temperature. After fungal growth and toxin production, rice was dried by using an oven at 70 °C; and finally, its powder was obtained. Qualitative and quantitative aflatoxin content in rice powder was measured by HPLC (Waters Alliance e2695 equipped with 2475 fluorescence detector, Milford, MA). In order to prepare the experimental diets, rice powder with a certain composition and level of aflatoxin was added and mixed to the basal diet up to a concentration of 0.5 mg/kg of AFB. According to the aflatoxin limit in poultry diets (0.02 mg/kg of feed), contamination (AFB) of the basal diet was 25 times of the extent permitted (Bhatti et al. 2016).

OTA was obtained by contamination of maize with Aspergillus ochraceus (NRRL 3174). Conidiospores of A. ochraceus were cultivated on potato dextrose agar substrates, for 5 d at 27 °C. Samples (100 g) of the broken maize were taken in 500 mL glass bottles and soaked in 50 mL tap water for 1 h. After 1 h, the bottles were capped and autoclaved. Then, the maize was inoculated with cultures of mould and incubated for 10 d, during which time full growth of the mould was achieved and OTA production took place. The maize with cultures of A. ochraceus was kept at the temperature of 25 °C during contamination. After 10 d, the maize was autoclaved and dried at 105 °C in a laboratory drying cabinet, ground and used for contamination of experimental premixes. HPLC analysis of contaminated maize revealed an OTA concentration of...
150 mg/kg. This maize was used for inoculation of the broiler diet at a final OTA concentration of 0.25 mg/kg. The entire diet preparation with OTA was carried out with due precautionary measures and utilisation of all protective equipment.

**Essential oils**

Chamomile and Thymus vulgaris plants were collected and the leaves were separated from the flowers. The flowers were washed and dried then ground into powder. Then, 25 g of the flowers’ powder was extracted using 250 mL of 80% ethanol at room temperature by maceration overnight, with occasional stirring (Alipour et al. 2015). The extract was then filtered and the solvent was evaporated using a rotary evaporator (Bio-Equip RE-52-3-5; Shanghai Qingpu Huxi Instruments Factory, Shanghai, China) at 30 °C to obtain the dried CFE and TOE. The obtained oils were analysed by gas chromatography/mass spectrometry (GC–MS) using an Agilent 6890/5973 GC-MS (Hewlett-Packard, Palo Alto, CA) according to Vasudeva and Sharma (2012), while the phenolic compounds were obtained according to Park et al. (2012) and identified by high performance liquid chromatography using an Agilent 1100 HPLC (Hewlett-Packard, Palo Alto, CA) according to Ozkan and Ozcan (2014) (Table 1).

**Experimental design, birds and diets**

In this study, a total of 720 one-day-old male chicks (Ross 308) with an average BW of 43.5 ± 0.5 g were used in a 28-day experiment. The chicks were assigned to 12 treatments, six replications and 10 chicks in each replication and were fed with experimental diet from 0 to 28 days of age. The experimental periods were a starter period (0–28 d). Basal diets were formulated according to the nutrient requirements for Ross 308 broilers (Aviagen 2007; Table 2). The basal diets were analysed for DM content (method 942.05; AOAC 2005), CP (method 920.32, AOAC 2005) by the Kjeldahl method (Kjeltac 2300 Nitrogen Analyser; Foss Tectar AB, Hoeganaes, Sweden) and EE (method 920.32, AOAC 2005) by a 1043 Soxtec HT system. The experiment was conducted as a 3 × 4 factorial arrangement to examine the effects of phytogenic additives (control, 3 CFE g/kg or 3 TOE g/kg), dietary contaminated diets (none, 0.50 mg/kg AFB, 0.25 OTA mg/kg or AFB + OTA) and their interactions. The experimental treatments were as follows: T1, control diet (CON); T2, CON + 0.50 mg/kg diet of AFB; T3, CON + 0.25 mg/kg diet of OTA; T4, CON + 0.50 mg/kg diet of AFB + 0.25 mg/kg diet of OTA; T5, CON + 3 g/kg diet of CFE; T6, CON + 3 g/kg diet of CFE + 0.50 mg/kg diet of AFB; T7, CON + 3 g/kg diet of CFE + 0.25 mg/kg diet of OTA; T8, CON + 3 g/kg diet of CFE + 0.50 mg/kg diet of AFB + 0.25 mg/kg diet of OTA; T9, CON + 3 g/kg diet of TOE; T10, CON + 3 g/kg diet of TOE + 0.50 mg/kg diet of AFB; T11, CON + 3 g/kg diet of TOE + 0.25 mg/kg diet of OTA; T12, CON + 3 g/kg diet of TOE + 0.50 mg/kg diet of AFB + 0.25 mg/kg diet of OTA.

Mash as physical feed form was used during the experiment. Each pen was equipped with tube feeders, automatic waterers and rice hulls. The pens

**Table 1. Chemical composition of chamomile flower (Matricaria chamomilla L.) extract (CFE), thyme-oil extract (TOE).**

| Compound     | Composition (%) |
|--------------|----------------|
| CFE          |                |
| Chamazulene  | 6.40           |
| Spiroether   | 5.10           |
| Bisabolol    | 6.30           |
| β-Farnesene  | 29.80          |
| α-Farnesene  | 9.30           |
| Germacrene   | 6.20           |
| TOE          |                |
| Carene       | 3.76           |
| para-Cymene  | 8.41           |
| gamma-Terpine| 30.90          |
| Terpineol    | 0.47           |
| Thymol       | 47.59          |
| Caryophyllene| 2.68           |

**Table 2. Ingredients and compositions of the basal diets (as-fed basis).**

| Item                  | Starter (0–10 d) | Grower (11–28 d) |
|-----------------------|------------------|------------------|
| Ingredients, %        |                  |                  |
| Maize                 | 49.30            | 59.60            |
| Wheat                 | 5.31             | 5.00             |
| Soybean meal, CP, 48% | 26.86            | 16.05            |
| Maize gluten          | 10.00            | 11.48            |
| Soybean oil           | 3.50             | 3.34             |
| Limestone             | 1.45             | 1.23             |
| Dicalcium phosphate   | 1.95             | 1.80             |
| Salt                  | 0.36             | 0.36             |
| α-Met                 | 0.52             | 0.58             |
| L-Lys                 | 0.25             | 0.06             |
| Vitamin-mineral premixa | 0.50          | 0.50             |

| Calculated composition, % |                  |
|---------------------------|------------------|
| ME, kcal/kg               | 3010.00          | 3150.00          |
| CP                         | 22.60            | 20.11            |
| Calcium                   | 1.00             | 0.90             |
| Available phosphorus      | 0.50             | 0.45             |
| Lys                       | 1.41             | 1.16             |
| Met                       | 0.68             | 0.52             |
| Met + Cys                 | 1.09             | 0.81             |

| Analysed composition, %   |                  |
|---------------------------|------------------|
| DM                        | 94.20            | 95.11            |
| CP                         | 22.62            | 20.56            |
| Fat                       | 5.27             | 5.76             |

*Vitamin and mineral premix supplied the following (per kilogram of diet): vitamin A (from vitamin A acetate), 10,000 IU; vitamin D3, 9790 IU; vitamin E (α-α-tocopheryl acetate), 30 IU; vitamin B12, 20 μg; riboflavin, 4.4 mg; calcium pantothenate, 40 mg; niacin, 22 mg; choline, 840 mg; biotin, 30 μg; thiamine, 4 mg; zinc sulphate, 60 mg; copper sulphate, 100 μg; selenium (sodium selenate), 0.2 mg; iodine, 1 mg; manganese oxide, 60 mg. ME: metabolizable energy; CP: crude protein; DM: dry matter.
had dimensions of 1.2 m length \times 0.85 m width \times 0.7 m height. Feed and water were supplied *ad libitum* throughout the experiment, and maintained on a 23-h lighting programme. The temperature was maintained at 32±1°C on day 1, and was gradually reduced to 22°C by 21 d of age.

**Performance data**

Feed intake and body weight were recorded on pen basis at the day of hatch, 11 and 28 days. The data were used to calculate average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) in each period and cumulatively.

**Blood collection and analyses**

At 28 d of age (end of the experiment), a 4-mL blood sample was obtained from the wing vein of two broilers in each replicate (12 samples per treatment), and then was collected into two tubes (2 mL in each tube). The first tube contained heparin as the anticoagulant, then centrifuged at 5000\times g for 10 min at 4°C. The collected plasma was stored at −20°C until further analysis. The plasma IgA, IgG and IgM concentrations were measured using an enzyme-linked immunosorbent assay ELISA kit from Bethyl Laboratories (Montgomery, TX). The ELISA procedure was carried out according to the protocol of the manufacturer and absorbance was measured at 450 nm.

The blood samples in the second tube (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) were placed at room temperature for 2 h for serum separation. Samples for serum analysis were then centrifuged at 3000\times g for 10 minutes. The serum without supernatant was removed into vials and immediately delivered to the laboratory for further biochemical analyses. Total cholesterol (Tch), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides (TGs) concentrations in the serum samples were analysed with an autoanalyzer (Autolab, BT 3500, Autoanalyzer Medical System, Rome, Italy) and were measured using reagent kits (Wako Pure Chemical Industries, Osaka, Japan).

**Relative organs weight**

After blood collection, the same bird was weighed individually and anaesthetised with carbon dioxide and killed by cervical dislocation. Broiler organs including the crop, gizzard, liver and pancreas were weighed and expressed as a percentage of body weight.

**Jejunum morphology**

The method of jejunum morphology measurement was based on Nourmohammadi and Afzali (2013). Fragments (approximately 3 cm in length) were harvested from the middle of the jejunum (i.e. between the distal portion of the duodenal loop and Meckel’s diverticulum). These samples were washed with cold PBS and fixed in 10% neutral buffered formalin at 4°C for morphometric analysis. Then, each fragment was embedded in paraffin, a 7-μm section of each sample was placed onto a glass slide and stained with alcian blue/haematoxylin and eosin for examination with a light microscope. Villous height (VH) and crypt depth (CD) of the jejunum were measured at a magnification ×100 using computer software (Sigma Scan, Jandel Scientific, San Rafael, CA). Then villous height to crypt depth ratio (VH/CD) was calculated. The height of 30 villous and the depth of 30 crypts were measured from each replicate, and the means were analysed statistically.

**Immune response**

The birds were vaccinated against Newcastle disease virus (NDV; strain *Viscerotropic velogenic*) at 1 (by oral-spray), 7 and 20 d of age (oral route by drinking water). At 17 and 28 d of age, blood samples were collected via cardiac puncture from 12 broilers selected randomly from each treatment to determine the NDV antibody titres by haemagglutination-inhibition test according to Alexander (1988). Serum was separated by centrifugation (1300 rpm for 15 min) after 1 h incubation at room temperature and stored at −20°C until the analysis.

**Statistical analysis**

In this experiment, pen was considered as experimental unit and data were analysed as a completely randomised design with 3×4 factorial treatment arrangements by using the GLM procedure of SAS (SAS Institute, Cary, NC) (SAS 2001). Percentage data were transformed by using arcsin transformation. Organ weights were analysed by analysis of covariance by using the GLM procedure of SAS (SAS Institute, Cary, NC) (SAS 2001) with BW as the covariate. Level of significance was set at 5% and when a significant effect
was indicated, treatment means were separated using Tukey-Kramer's test. The statistical model was as follows:

\[ Y_{ij} = \mu + D_i + CH_j + (D \times CH)_{ij} + e_{ij} \]

where \( Y_{ij} \) is the individual observation; \( \mu \) is the overall mean; \( D_i \) is the diet effect; \( CH_j \) is the contaminant effect; \( (D \times CH)_{ij} \) is the interaction between diet and contaminant effect; \( e_{ij} \) is the error term with mean 0 and variance \( \sigma_e^2 \).

**Results**

As shown in Table 3, there were no significant differences in the growth performance data in the starter period (d 0–10). During the grower (d 11–24) and overall (d 0–28) periods, broilers fed CFE and TOE supplemented diets had higher (p < .05) ADFI and ADG, and lower FCR (p < .01) than those fed CON diet. When compared with the control, the OTA-AFB combination decreased ADG (p < .01) at the same time. Compared with the CON diet, the consumption of contaminated feeds (AFB, OTA and AFB+OTA) resulted in a significant reduction in ADFI and feed efficiency during the grower and overall periods (p < .01). Significantly increased ADG and ADFI during the overall period were observed in the two essential oil-treated groups when compared with OTA-AFB-contaminated group (p < .05).

The effects of dietary essential oils on the gut morphology of broilers treated with AFB and OTA are given in Table 4. Inclusion of CFE and TOE had no significant effects on relative weight of digestive organs (crop, gizzard, liver and intestine) and jejunum morphology. The relative weights of gizzard (p = .011), liver (p = .004) and pancreas (p = .024) of OTA-treated chickens were higher than those fed CON diets. Jejunal VH and VH/CD ratio were significantly decreased in broilers fed with OTA, AFB and OTA+AFB contaminated diets as compared to those fed CON diet (p < .001).

The supplenations of CFE and TOE to diet significantly increased the concentration of Tch (p = .003) and HDL (p = .012), but did not affect the TG and LDL levels in serum (Table 5). As shown in Table 5, dietary CFE and TOE supplementation had significant effect on plasma immunoglobulin levels. Inclusion of CFE and TOE resulted in an increase (p < .001) in the plasma IgG and IgM levels compared with the CON diet, but did not affect the IgA concentration. On day 28, serum TG, Tch and HDL concentrations were decreased in birds fed the diets contaminated with

Table 3. Effects of dietary chamomile flower (Matricaria chamomilla L.) extract (CFE), thyme-oil extract (TOE), aflatoxin B1 (AFB) and ochratoxin A (OTA) on growth performance of broilers.

| Treatment | Final BW | ADFI, g | ADG, g | FCR | ADFI, g | ADG, g | FCR | ADFI, g | ADG, g | FCR |
|-----------|---------|--------|-------|-----|--------|-------|-----|--------|-------|-----|
| Control (CON) | 1437.000 | 28.300 | 23.000 | 1.230 | 95.200 | 64.300 | 1.180 | 71.300 | 49.600 | 1.440 |
| CON + 0.50 AFB, mg/kg | 1377.000 | 27.600 | 22.900 | 1.210 | 89.400 | 61.200 | 1.460 | 67.300 | 47.500 | 1.410 |
| CON + 0.25 OTA, mg/kg | 1372.000 | 29.000 | 23.100 | 1.260 | 88.500 | 60.700 | 1.450 | 67.200 | 47.300 | 1.420 |
| CON + AFB + OTA | 1399.000 | 28.700 | 23.400 | 1.230 | 88.700 | 62.200 | 1.430 | 67.300 | 48.300 | 1.390 |
| 3 CFE, g/kg | 1670.000 | 30.200 | 25.400 | 1.190 | 99.700 | 76.200 | 1.310 | 74.900 | 58.000 | 1.290 |
| 3 CFE, g/kg + 0.50 AFB, mg/kg | 1565.000 | 29.100 | 25.300 | 1.150 | 93.300 | 70.000 | 1.320 | 70.400 | 54.200 | 1.300 |
| 3 CFE, g/kg + 0.25 OTA, mg/kg | 1553.000 | 30.400 | 25.600 | 1.190 | 91.700 | 69.500 | 1.320 | 69.800 | 53.800 | 1.300 |
| 3 CFE, g/kg + AFB + OTA | 1528.000 | 29.800 | 25.300 | 1.180 | 90.500 | 68.300 | 1.330 | 68.800 | 52.900 | 1.300 |
| 3 TOE, g/kg | 1670.000 | 30.000 | 24.700 | 1.210 | 99.200 | 76.500 | 1.300 | 74.900 | 58.000 | 1.280 |
| 3 TOE, g/kg + 0.50 AFB, mg/kg | 1520.000 | 28.700 | 24.600 | 1.170 | 92.700 | 68.300 | 1.360 | 69.800 | 52.600 | 1.330 |
| 3 TOE, g/kg + 0.25 OTA, mg/kg | 1540.000 | 28.900 | 24.800 | 1.160 | 92.400 | 69.100 | 1.340 | 69.700 | 53.300 | 1.310 |
| 3 TOE, g/kg + AFB + OTA | 1457.000 | 29.500 | 24.900 | 1.180 | 89.300 | 64.400 | 1.360 | 67.900 | 50.300 | 1.350 |
| SEM | 2.400 | 0.270 | 0.240 | 0.010 | 1.630 | 1.180 | 0.075 | 1.060 | 0.970 | 0.031 |

AFB + OTA; 0.50 AFB mg/kg + 0.25 OTA mg/kg; ADFI: average daily feed intake; ADG: average daily gain; FCR: feed conversion ratio. a,b,cMeans in the same column without the same superscript differ significantly (p < .05).
mycotoxins compared with the CON diet. A significant decrease ($p < .001$) in plasma immunoglobulin levels (IgA, IgG and IgM) was found in the chickens given dietary concentrations of toxins (Table 5).

At 17 d of age, there was no significant difference of anti-NDV antibody titre among the groups (Table 6). At 28 d of age, anti-NDV antibody titre was higher ($p < .001$) for broilers fed essential oils-supplemented diets than for broilers fed the CON diet, but lower ($p < .001$) from the OTA-AFB-contaminated diets than for those fed the CON diet.

**Discussion**

**Effect of mycotoxins**

Both OTA and AFB are important mycotoxins due to their toxicity and their occurrence in the contaminated diets in Iran. Numerous reports on effects of mycotoxins on broiler performance have been previously reviewed by Abidin et al. (2011). There is a general belief that dietary AFB and OTA reduce weight gain, feed intake and increase FCR. The addition of low doses of mycotoxin (0.50 mg/kg AFB, 0.25 mg/kg OTA and AFB + OTA) to maize-soybean diets decreased the growth performance of the broiler chicks. In other studies, in which diets were contaminated with lower amounts of AFB and OTA (0.20 mg/kg) (Santin et al. 2006; Sawarkar et al. 2011), isolated or in combination, differences in growth performance were not observed due to the low doses of mycotoxin used. Other studies reported a detrimental effect on body weight and feed consumption when broilers were fed AFB and OTA mixed at levels of 0.10 mg/kg or higher (0.40 and 0.60 mg/kg AFB) (Anand et al. 2008; Sawarkar et al. 2011). The adverse effects of OTA and AFB on growth

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**Table 4.** Effects of dietary chamomile flower (*Matricaria chamomilla* L.) extract (CFE), thyme-oil extract (TOE), aflatoxin B1 (AFB) and ochratoxin A (OTA) on gut morphology of broilers.

| Main effects | Crop | Gizzard | Liver | Pancreas | Jejunum morphology, μm |
|--------------|------|---------|-------|----------|------------------------|
| Diet         |      |         |       |          | VH | CD | VH/CD |
| Control (CON) | 0.3220 | 2.6520 | 2.5330 | 0.3250 | 867.0000 | 292.0000 | 2.9700 |
| CON + 3 CFE, g/kg | 0.3150 | 2.2650 | 2.3550 | 0.2750 | 874.0000 | 289.0000 | 3.0200 |
| CON + 3 TOE, g/kg | 0.3210 | 2.2800 | 2.3780 | 0.2630 | 880.0000 | 288.0000 | 3.0500 |
| Challenge    |      |         |       |          |    |    |      |
| No challenge | 0.3220 | 1.8870b | 2.0670b | 0.2170b | 927.0000a | 282.0000 | 3.2900a |
| 0.50 AFB, mg/kg | 0.3240 | 2.3500ab | 2.5300ab | 0.2730b | 854.0000b | 292.0000 | 2.9300b |
| 0.25 OTA, mg/kg | 0.3160 | 2.6730a | 2.6570a | 0.3200a | 888.0000b | 291.0000 | 3.0000b |
| AFB + OTA     | 0.3170 | 2.6870a | 2.7330a | 0.3410a | 911.0000c | 294.0000 | 2.7500c |
| SEM           | 0.0035 | 0.0149 | 0.0167 | 0.0024 | 17.4.0000 | 4.5000 | 0.1240 |

NH: villous height; CD: crypt depth; VH/CD: villous height-to-crypt depth ratio.

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**Table 5.** Effects of dietary chamomile flower (*Matricaria chamomilla* L.) extract (CFE), thyme-oil extract (TOE), aflatoxin B1 (AFB) and ochratoxin A (OTA) on blood parameters and humeral immune.

| Main effects | Lipid blood parameters, mg/dL | Plasma immunoglobulin |
|--------------|-------------------------------|-----------------------|
| Lipid        | TG | Tch | LDL | HDL | IgA | IgG | IgM |
| Diet         |    |    |    |    |    |    |    |
| Control (CON) | 76.10 | 155.00b | 61.40 | 70.10b | 38.87 | 2.44b | 180.67b |
| CON + 3 CFE, g/kg | 78.60 | 170.00a | 64.20 | 83.60a | 42.20 | 3.37a | 187.30a |
| CON + 3 TOE, g/kg | 80.30 | 171.00a | 64.00 | 83.80a | 43.64 | 3.69a | 191.49a |
| Challenge    |    |    |    |    |    |    |    |
| No challenge | 89.90a | 179.00a | 67.00 | 89.10a | 53.79a | 4.84a | 212.74a |
| 0.50 AFB, mg/kg | 77.50b | 157.00b | 61.30 | 72.70b | 42.70b | 2.27b | 187.27b |
| 0.25 OTA, mg/kg | 72.60b | 164.00b | 61.90 | 79.00b | 32.71b | 2.19b | 172.25b |
| AFB + OTA     | 73.40b | 162.00b | 62.80 | 75.80b | 37.03b | 2.03b | 173.68b |
| SEM           | 1.15 | 1.80 | 1.32 | 1.08 | 0.67 | 0.03 | 1.71 |

TGs: triglycerides; Tch: total cholesterol; LDL: low density lipoprotein; HDL: high density lipoprotein.

AFB + OTA: 0.50 AFB mg/kg + 0.25 OTA mg/kg.

Means in the same column without the same superscript differ significantly ($p < .05$).
performance have been related with a decrease in protein and energy utilisation, probably as a consequence of a deterioration of the digestive and metabolic efficiency of the birds (Valtcchev et al. 2015). The results imply that the concentration of the combined toxins be critical to their effects on the growth performance of the chickens.

The weights of gizzard, liver and pancreas relative to body weight of broilers have been reported to be affected by AFB. The relative weight of these organs has been noted to decrease by some authors (Kubena et al. 1998; Qu et al. 2017), while to increase by other has been noted to decrease by some authors (Kubena et al. 1998; Qu et al. 2017). The relative weight of these organs to body weight of broilers have been reported to be different in the aforementioned studies. It further supports the notion that the combined toxins can be critical to their effects.

Maintenance of normal microarchitecture in the small intestine is very important for proper growth and development (Nourmohammadi and Afzali 2013). In the current research, the addition of mycotoxin (OTA, AFB and OTA + AFB) to diets decreased VH and VH/CD in the jejunum. This effect was greater with the AFB + OTA combination. The decreases in VH and VH/CD of the jejunum in OTA-AFB-fed chicks indicate the unit absorptive surface of small intestine would deteriorate during a chronic exposure to low levels of mycotoxin. This effect may be explained by the results of Shen et al. (1994), who demonstrated that epithelial shedding may be related to the oxidative damage which might be one of the underlining mechanisms for mycotoxin-induced cell injury and DNA damage.

The results of the present study are in agreement with that from Ji et al. (2016) and Shang et al. (2016), who revealed that mycotoxin decreased lipid blood parameters. Decreased cholesterol levels may be attributed to the hepatotoxic effects of AFB and OTA or their synergetic effect that is characterised by impairment of transport and lipid metabolism of liver (Shang et al. 2016). The HDL can remove cholesterol from the blood vessels and carries it back to the liver, where it can be processed and sent out of the body. The liver is an important place for synthesis of HDL. The reduced HDL may suggest that mycotoxins exert a toxic effect on liver of the broilers.

Early researches have found that mycotoxin could cause a significant inhibition of development, lymphoid depletion, and a decrease of relative weight of bursa of Fabricius (Khatke et al. 2013). Thus, the significant decrease in the plasma IgA, IgM and IgG concentrations observed in this research experiment can be explained by the retarded growth of the bursa of Fabricius. Furthermore, the decreased IgA production observed in this study may be related to the reduction of T lymphocytes as well as cytokines induced by AFB and OTA. It is well demonstrated that broilers fed with the dosage of 0.3 mg/kg AFB diet showed a decline population of T cells in the peripheral blood and decreases of mature T cell number and some cytokine (IL-2 and IL-6) mRNA expression in the ileum (Shang et al. 2016).

The results of the present study showed that OTA and AFB toxins, alone or in combination, decreased the level of anti-NDV serum antibody titre, indicating that mycotoxins impaired humoral-mediated immunity. Xue et al. (2010) demonstrated that a significant decrease in anti-NDV antibody titre was recorded in the broilers given 0.25 mg/kg of OTA or a combination of T-2 toxin (0.50 mg/kg) and OTA. Although the previous study focussed on different toxin effects on different immune responses, it is commonly accepted that mycotoxins could impair immune function greatly. The results imply that OTA-AFB contaminated diets impaired the production of anti-NDV antibodies.

**Table 6. Effects of dietary chamomile flower (*Matricaria chamomilla* L) extract (CFE), thyme-oil extract (TOE), aflatoxin B$_1$ (AFB) and ochratoxin A (OTA) on antibody titres against Newcastle disease virus of broilers.**

| Main effects | Log$_2$ titer$^a$ | Diet | Challenge | p Value | SEM | p | Diet | Challenge | Diet x Challenge |
|-------------|-------------------|------|-----------|---------|-----|---|------|-----------|-----------------|
| 17 d        |                   |      |           |         |     |   |      |           |                 |
| Control (CON) | 2.930             | 3.420$^b$ | 2.910     | 3.640$^b$ | 0.032 | .341 | <.001 | .185 | .995 |
| CON + 3 CFE, g/kg | 2.910             | 3.670$^b$ | 3.390$^a$ |        |      |   |      |           |                 |
| CON + 3 TOE, g/kg | 2.920             | 3.390$^a$ | 3.050$^b$ |        |      |   |      |           |                 |
| AFB          |                   |      |           |         |     |   |      |           |                 |
| No challenge | 2.910             | 3.670$^b$ | 3.390$^a$ |        |      |   |      |           |                 |
| 0.50 AFB, mg/kg | 2.950             | 3.070$^b$ | 3.050$^b$ |        |      |   |      |           |                 |
| 0.25 OTA, mg/kg | 2.940             | 3.070$^b$ | 3.050$^b$ |        |      |   |      |           |                 |
| AFB + OTA    |                   |      |           |         |     |   |      |           |                 |
| No challenge | 2.920             | 3.030$^b$ | 3.050$^b$ |        |      |   |      |           |                 |
| 0.25 OTA mg/kg | 2.920             | 3.070$^b$ | 3.050$^b$ |        |      |   |      |           |                 |

$^a$Means in the same column without the same superscript differ significantly (p < .05).

$^b$Antibody titres were subjected to log$_2$ transformation.

AFB + OTA: 0.50 AFB mg/kg + 0.25 OTA mg/kg.
in the serum and essential oils were unable to reduce the toxicity.

**Effect of essential oils**

Essential oils (TOE and CFE) counteracted the toxic effect, which corroborates the findings of other studies (Manafi et al. 2014; El-Khoury et al. 2017). Esper et al. (2014) demonstrated that monoterpenes are effective against yeasts and filamentous fungi. The components of essential oils are also effective against moulds of the genus *Aspergillus*, including *A. fumigatus*, which is the most frequent cause of aspergillosis in poultry. The study by Esper et al. (2014) also suggests that the oil of oregano (*Origanum vulgare*) may serve as a protective feed supplementation against AFB.

Inclusion of EO in poultry feed did not improve growth performance during the starter phase. This could be due to digestive enzyme secretion capacity, which is reported to be relatively low in young chicks, only increasing toward d 21 (Khattak et al. 2014). Improved performance parameters during the grower and finisher phase could be attributed to the presence of EO in diet, which encourages secretions of endogenous digestive enzymes, which then enhance nutrient digestion and gut passage rate in birds. The results of growth performance enabling us to verify the activity of these two essential oils in order to envision their potential use as antifungal and/or antiaflatoxicogenic.

In different studies conducted to evaluate the effect of EO on serum Tch, LDL and HDL levels contradictory results have been obtained. The increased in cholesterol concentration in the present study could be due to the phenolic compounds of carvacrol and thymol that exhibit considerable antimicrobial activity (Vasudeva and Sharma 2012) that may depress fat absorption due to bile acid deconjugation (El-Khoury et al. 2017).

Several herbs and EO have been reported as having immunomodulatory effects such as lymphocyte expression, phagocytosis, modulation of cytokine and immunoglobulin secretion, histamine release and so on (Chowdhury et al. 2018). It has been reported plant extracts have positive immune effects such as increase in lymphocyte proliferation rate, phagocytic rate as well as increase in immunoglobulins such as IgA and IgM in the blood of broiler chickens (Reis et al. 2018). It has also been documented that EO effectively reduces the pro-inflammatory cytokine production and thereby attenuates the 2,4,6-trinitrobenzenesulfonic acid-induced colitis in mice (Manafi et al. 2014). Nevertheless, the mechanisms mediating the suppressive effects of EO on inflammation are still unknown.

**Conclusions**

The results of this study indicate that AFB and OTA, alone or in combination, caused negative responses for performance and immune function even at combined concentrations as low as 0.25 mg/kg diet of OTA and 0.50 mg/kg diet of AFB. Dietary inclusion of 3 g/kg diet of TOE and 3 g/kg diet of CFE had positive effects on growth performance and immune system of broiler chicks. It was concluded that the inclusion of TOE and CFE can be an alternative to ameliorate the adverse effects of low doses of AFB and OTA in broiler diets.

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