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The effect of earthworm (*Eisenia foetida*) meal with vermi-humus on growth performance, hematology, immunity, intestinal microbiota, carcass characteristics, and meat quality of broiler chickens

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ABSTRACT

The present investigation was aimed to evaluate the effect of varied amount of earthworm meal (EW) and vermi-humus (VH) on the growth performance of broiler chickens. Three hundred 1-d-old broiler chickens were assigned to 5 starter treatments with 5 pens per treatment, and 12 broiler chickens per pen in a completely randomized design from d 0–14 of the study. Dietary treatments were [per kilogram dry matter (DM)]: control (0 g EW and 0 g VH/kg of DM), and the diets containing 10 g VH/kg of DM supplemented with 0, 10, 20, or 30 g EW/kg of DM. At the end of the study (d 42), one representative broiler chicken per pen, close to the average body weight, was selected for blood sampling using a sterile needle and heparinized vacuum tube. The outcomes of the study depicted the greater overall feed intake value in broiler chicken fed the control diet than those fed the diets containing VH or EW or both, and it decreased linearly and quadratically ($P < 0.05$) as the amount of EW supplementation increased. The average weight gain for the chickens was numerically increased as supplementation of EW was increased (linear, $=0.3$; quadratic $P=0.4$). On the other hand, overall feed conversation ratio was slightly greater ($P=0.02$) in broiler chickens fed the control diet, and it decreased linearly ($P=0.03$) as dietary EW supplementation increased. Additionally, the serum total protein, albumin, Ca, and P concentrations were lower in broiler chickens fed the control diet, and those variables increased linearly ($P < 0.05$) as dietary EW increased. In like manner, humoral immune response (except heterophil/lymphocyte ratio) and relative weights of immune organs were lower in broiler chickens fed the control diet. Remarkable differences were observed between carcass and ileum characteristics of broiler chickens under treatments. Varied concentrations of EW showed increased total counts of lactic acid bacteria (linear, $P < 0.05$; quadratic, $P=0.3$) and reduced population of pathogenic intestinal microbiota (linear, $P < 0.05$; quadratic, $P > 0.05$). Similarly, the meat quality of broiler chicken was markedly affected linearly ($P < 0.05$) by the supplementation of increased dietary EW. Briefly, diets containing 30 g EW/kg of DM can positively affect the growth performance of broiler chickens and produce meat with better characteristics.

1. Introduction

Globally, the broiler chickens/poultry industries are important sources of animal protein for humans with increasing per capita consumption of broiler chicken meat. The growth in poultry production is having a profound influence on the demand for feed and raw materials. Feed is the costliest input for poultry production, with increasing necessity for low-priced and high-quality feeds to remain competitive and continue to grow to meet the demand for animal protein (Leeson and Summers, 2005).

At present, there is a growing demand of animal protein for human consumption due to remarkable increment in the world's population.

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Moreover, there is a wide gap between supply and demand for traditional ingredients and is expected to widen over the coming decades, providing a compelling reason for exploring the usefulness of unconventional feedstuffs in feed formulations. In the current scenario, developing countries are undertaking considerable efforts in order to utilize diversified sources of feed ingredients, in particular protein materials. Soybean meal, fish meal and meat meal have been used as the key sources of protein for poultry diets globally. Unfortunately, despite the main protein sources, these feed ingredients are costly and lack worldwide availability for animal feed formulation. Therefore, there is an increasing concern regarding the exploitation of alternative animal proteins feedstuffs. In fact, there are some alternatives that can be utilised as substitute for soybean meal, fish meal and meat meal in poultry diets. One such potential alternative is earthworm meal (EW; Bahadori et al., 2015; Rezaeipour et al., 2014).

Earthworm (*Eisenia foetida*) meal has many interesting characteristics viz. the high reproduction rate of the annelid, and the ability to feed and grow the worms on a wide range of organic residues. Additionally, the meal is easy to be processed and stored (Rodriguez-Campos et al., 2014). Furthermore, EW is superior to fish meal in terms of protein quality, constituting essential amino acids, adequate amount of fatty acid and omega 3 (Bahadori et al., 2015). Loh et al. (2005) reported that EW contains 580–710 g/kg of CP (DM basis) and high lysine concentrations. Lysine requirement in poultry is relatively high; therefore, EW would be a suitable feed ingredient in order to meet the lysine requirement of poultry (Vielma et al., 2003; Istiqomah et al., 2009).

Vermi-humus (VH) is a source of humic acid, resulting from the decomposition of organic materials in soil with a high biological value as a feed additive (Rezaeipour et al., 2014). Humic acid has the ability to inhibit bacterial and fungal growth, thereby decreasing the level of mycotoxin in feed. Enhancing stress management and immune system, and preventing intestinal diseases, mainly diarrhea in animals is described as its beneficial effects. Additionally, the humic acid in feed had improved not only gut health for better nutrient utilization but also the health status by developing immunity (Rath et al., 2006). Mixing EW and VH is a significant approach towards improving the feed efficiency in poultry feeding (Bahadori et al., 2015) due to the improved intestinal microbiota profile (Husseiny et al., 2008) and high protein content (Vielma et al., 2003). However, the valuable information on the exploitation of EW and VH as alternative animal feed ingredients and their impact on animal growth performance are scanty (Bahadori et al., 2015). Therefore, the major objective of the present context was to determine the effect of different amounts of EW and VH (during the constant period) on growth performance, hematology, immunity, microbiota, ileum morphology, carcass characteristics, and meat quality of broiler chickens.

2. Materials and methods

2.1. Experiments and test items

The experiments were conducted at the Agricultural and Natural Resources Research Center of Ilam, Iran. The handling and treatments of broiler chickens were approved by the Ethic Committee of Sanandaj Branch, Islamic Azad University, Sanandaj. Earthworm meal and VH were obtained from a local supplier (Amizeh Tabiaat Co, Tehran, Iran). The chemical composition and amino acid profiles of EW and VH are shown in Table 1.

2.2. Broiler chickens, feeding, and management

Three hundred 1-d-old broiler chickens (Ross308; Aviagen, Newbridge, Scotland, UK) with 43.0 ± 1.3 g of body weight were randomly assigned to 5 treatments with 5 pens per treatment and 12 broiler chickens per pen in a completely randomized design. The treatments were: a basal diet without addition of EW or VH (control), and the basal diet containing VH or EW at varied concentration as a starter diet from d 0–14 of the study. From d 14–28, the broiler chickens were fed a grower diet, while, from d 28–42 the broiler chickens were fed a finisher diet. The ingredients and composition of the diets (starter, grower, and finisher diets) are presented in Table 2. Diets were formulated to meet the nutritional requirements for the strain of broiler chickens (Aviagen, 2007). During the first 10 d of rearing, feed was available on plastic trays, and then feed was available in appropriate feeders. Broiler chickens had free access to diets and clean fresh water during the complete experimental period. The EW, VH, and experimental diets were ground to pass through a 1-mm screen using a Wiley mill (Arthur H. Thomas, Philadelphia, PA, US.), and then sent for chemical analysis (Evonik Industries, Rellinghauser Straße, Essen, Germany).

Table 1
Chemical composition and amino acid profiles of earthworm (*E. foetida*) meal and vermi-humus.

Item	Earthworm meal	Vermi-humus
Chemical composition (g/kg dry matter)		
Dry mater (g/kg fresh weight)	910.0	–
Organic matter	–	350.9
Crude protein	656.8	72.7
Metabolizable energy (MJ/kg)	13.6	–
Fat	70.3	1.4
Ca	4.5	89.7
P	12.2	7.0
Humic acid	–	18.6
Folicacid	–	> 10
Amino acid (g/kg dry matter)		
Met	12.0	–
Cys	9.5	–
Met + Cys	21.5	–
Ly	44.4	–
Thr	29.9	–
Arg	44.1	–
Ile	29.5	–
Leu	50.2	–
Val	32.2	–
His	17.4	–
Phe	27.2	–
Gly	34.6	–
Ser	29.4	–
Pro	24.1	–
Ala	34.4	–
Asn	65.4	–
Gln	87.6	–

Ambient temperature was controlled at 32 °C in broiler chicken's place, then reduced periodically to 24 °C at 3 weeks of age, and further maintained at 24 °C until d 42 with relative humidity about 50–65%. During the whole experiment, light was on for 24 h/d.

Broiler chickens were vaccinated against Avian Influenza (AI) at d 7, infectious bronchitis virus at d 0, Newcastle disease (ND) virus at d 7, 18, and 26; and Gumboro virus at d 16, and 21. To reduce the stress caused by vaccination, a multi-electrolyte solution was used in the drinking water for 24 h before and after vaccination.

Humoral immune response of broiler chickens to the Newcastle vaccine (d 18 and 28) and influenza (d 21 and 28) was studied based on hemagglutination inhibition (HI) test. It has been reported that HI test is an excellent indicator of the immune status and disease resistance of a flock especially to assess protective response following vaccination (Collett, 2013; Miller and Koch, 2013). Sheep red blood cells (SRBC) were used as a test antigen to quantify specific antibody responses. On d 12, broiler chickens were injected with SRBC and sampled at d 24 and 32 to study the humoral immune response. For the SRBC injection, initially 1 mL of PBS along with 10 mL of SRBC was mixed, and 0.5 mL of the obtained solution was drawn into the syringe and injected under

Table 2
Ingredients and chemical composition of experimental diets[a] with different amounts of earthworm meal (EW) and vermi-humus (VH).

Item	Treatments (starter)[b]					Grower	Finisher
	Control	0EW-10VH	10EW-10VH	20EW-10VH	30EW-10VH		
Ingredients(g/kg dry matter)							
Earthworm meal	0	0	10.0	20.0	30.0	0.0	0
Vermi-humus	0	10.0	10.0	10.0	10.0	0	0
Corn	511.0	500.0	512.0	526.0	527.0	586.0	610.0
Corn gluten	70.0	70.0	70.0	70.0	70.0	0	0
Corn oil	13.3	17.0	13.3	9.5	7.3	24.3	34.3
Soybean meal	353.0	355.0	337.0	318.0	310.0	347.0	316.0
Met	2.5	2.5	2.5	2.4	2.3	2.7	2.3
Lys	3.9	3.9	3.9	3.8	3.5	1.7	1.1
Thr	0.7	0.7	0.7	0.6	0.4	0.6	0.4
Choline	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Digestible crude protein	20.0	19.7	19.4	19.0	18.6	17.7	16.2
$CaCO_3$	12.9	10.7	10.7	10.6	10.6	10.3	10.3
$NaHCO_3$	5.3	3.5	3.4	3.3	3.1	0	0
NaCl	1.0	1.0	1.0	1.0	1.1	3.4	3.4
Vitamin/mineral premix[c]	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Analyzed composition (g/kg dry matter)							
Crude fat	34.3	37.5	34.2	30.8	28.7	46.3	56.9
Crude fiber	36.2	36.1	35.1	34.0	33.5	35.5	33.7
Calculated metabolizable energy (MJ/kg)	12.2	12.5	12.5	12.5	12.5	12.6	13.0
Crude protein	242.8	243.1	243.1	243.1	243.1	203.7	191.8
Lys	13.4	13.4	13.4	13.4	13.4	11.2	10.0
Met	6.1	6.1	6.1	6.1	6.1	5.5	4.9
Met + Cys	9.6	9.6	9.6	9.6	9.6	8.3	7.6
Thr	8.4	8.4	8.4	8.4	8.4	7.2	6.6
Try	2.3	2.3	2.2	2.1	2.1	2.2	2.0
Arg	13.8	13.8	13.7	13.6	13.8	12.7	1.8
Ile	9.1	9.1	9.1	9.1	9.2	7.7	7.2
Val	10.0	10.0	10.0	10.1	10.2	8.5	8.0
Leu	21.7	21.7	21.7	21.8	22.0	15.9	15.1
Ca	10.3	10.3	10.3	10.3	10.3	8.8	8.4
P	4.9	4.9	4.9	4.9	4.9	4.4	4.1
Na	1.6	1.6	1.6	1.6	1.6	1.6	1.6
K	8.8	8.9	8.7	8.6	8.6	8.6	8.1
Cl	1.8	1.8	1.8	1.8	1.8	2.9	2.8
Dietary electrolyte balance (mEq/kg)	243.9	243.9	235.2	225.0	220.0	209.1	198.1
Linoleic acid	13.4	13.1	13.2	13.4	13.0	13.9	14.1

[a] Starter (d 0 to14); grower (d14 to 28); finisher (d 28 to 42)
[b] The treatments were: a basal diet without addition of EW or VH (0EW-0VH; control), and the diet containing 10 g VH/kg of DM supplemented with 0, 10, 20, or 30 g EW/kg of DM as a starter diet from d 0–14 of the study.
[c] Provides per kilogram of diet: 18,000 IU vitamin A, 4000 IU vitamin D_3, 36 mg vitamin E, 4 mg vitamin K_3, 3.5 mg thiamine, 13.2 mg riboflavin, 20 mg niacin, 19.6 mg D-pantothenic acid, 5.9 mg vitamin B_6, 2 mg folic acid, 30 μg vitamin B_{12}, 500 mg choline chloride, 200 mg Mn, 169.4 mg Zn, 100 mg Fe, 20 mg Cu, 2 mg I, and 0.4 mg Se.

the skin of the broiler chicken's breast. At 7 d post injection, all birds were bled by brachial venipuncture, and 3 mL of blood was collected for primary antibody response.

2.3. Blood chemistry

On d 42, 5 male broiler chickens per treatment that were close to the pen average body weight were selected for blood collection. Blood samples (1 mL/broiler chicken) were collected from the wing vein into heparinized tubes, and samples were centrifuged (13000 × *g* for 15 min at room temperature) within 2 h after collection. Plasma samples were stored in microcentrifuge tubes at −20 (degree) Celsius, until analyzing using specific kits (Pars Azmoon, Tehran, Iran). One hundred cells were counted and differentiated into heterophils, lymphocytes, and monocytes. Further, the mean heterophils/lymphocytes ratio was calculated.

2.4. Growth performance, carcass characteristics, and intestinal morphology

Feed intake was recorded weekly by pen. The body weight was recorded weekly per pen. Mean daily weight gain was calculated by the difference between 2 consecutive weighing. On d 42, 5 male broiler chickens per treatment that were close to the pen average body weight were selected for studying carcass and gastrointestinal segments. After slaughter and feather picking, the head and legs were removed. Broiler chickens were eviscerated. Weights of the carcass, gastrointestinal tract and limphoid organs (spleen, thymus, and bursa of Fabricius) were recorded.

Ileal segments of approximately 2 cm were taken from midway between Meckel's diverticulum and the ileocecal junction. These segments were immediately flushed twice with phosphate buffer saline (PBS) to remove luminal digesta. Tissue samples were fixed in 10 mL of fresh formalin buffer/L, dehydrated, cleared, and embedded in paraffin. Sections were cut to a thickness of 6 μm, placed on glass slides, stained with hematoxylin-eosin (Kiernan, 2008), and examined by light microscopy. Fifteen villi having a lamina propria were randomly selected on each slide. Villus height was defined as the distance from the tip to the base, excluding the intestinal crypt, and crypt depth was defined as the distance from the villus base to the muscularis layer, not including the intestinal muscularis. The villus height/crypt depth ratio was calculated.

2.5. Microbiota measurements

On d 42, 1 broiler chicken per pen (in total 5 broiler chickens per treatment) were selected for cecal contents collection. The cecum

contents were placed on agar plates for the determination of bacterial growth and colony counts. The culture media were prepared 24 h before collection as follows: Man Rogosa Sharpe (MRS) agar was used to culture *Lactobacilli*, Eosin Methylene Blue (EMB) agar to culture *E. coli*, and nutrient agar (NA) was used to culture total aerobic bacteria counts, as described by Abbasi et al. (2015).

2.6. Meat quality

Thiobarbituric acid reactive substances (TBARS) assay by measuring malondialdehyde (MDA), a product of oxidation during the storage period, was determined based on the method of Tarladgis et al. (1960) and used for the determination of MDA content as it was considered a standard method for MDA analysis. Briefly, each homogenized sample was added to 97.5 mL of distilled water and 2.5 mL of 6 N HCl and then distillated until reaching 200 mL of distillate. Five mL of thiobarbituric reactive reagent (0.02 M TBARS in 900 glacial acetic acid/L) was added in 5 mL of distillate. The mixture was incubated for 35 min in boiling water. After cooling, the absorbance was measured at 538 nm. The multiplication by 7.8 was used in order to calculate the distillation TBARS number as described by Tarladgis et al. (1960).

2.7. Statistical analysis

A completely randomized experimental design involving 5 treatments were subjected to statistical analysis of variance using the General Linear Model procedures of the SAS (SAS, 2000). Polynomial (linear and quadratic) contrasts were used to examine the dose responses due to increasing amounts of EW in the diet. When data were not normally distributed or when some of the required assumptions for ANOVA were violated, data were transformed before the analysis, following the guidelines as described by Büchse et al. (2007). Considering the nature and distribution of data for each variable, the most appropriate transformation was applied according to the criteria reported by Büchse et al. (2007). Thus, microbial counts were log-transformed. Square root transformation was applied to heterophils to lymphocytes ratio, to relative weights of immune organs (spleen, thymus, bursa of Fabricius), and to water holding capacity, whereas the arc sin transformation was used for carcass data (relative weights of liver, thigh, breast, abdominal fat), and relative weight of ileum.

3. Results

3.1. Feed intake, weight gain, and feed conversion ratio

During biweekly measurement intervals over the whole experimental period (d 0–42), the broiler chickens fed the diet containing 100 g EW/kg of DM showed higher feed intake (linear, $P=0.01$; quadratic, $P=0.04$) value in a comparison with control ($P=0.01$) as well as diets supplemented with varied amounts of EW (linear, $P=0.01$; quadratic, $P=0.04$) (Table 3). The average daily weight gain during measurement intervals and during the whole experimental period of 42 d revealed no remarkable variations among the broilers fed control diet ($P > 0.05$) as well as EW supplementation (linear, $P > 0.05$; quadratic, $P > 0.05$). Feed conversion ratio was not affected in the starter and grower phases, but during the finisher phase and for the overall experiment (from day 1–42) feed conversion decreased linearly as the level of EW supplementation was increased.

3.2. Blood biochemical measurements

Table 4 shows the blood biochemical measurements of broiler chickens supplemented with increased amounts of EW. Total protein, albumin, Ca, and P contents were found to be increased in the broiler chickens fed with various amounts of EW supplementation (linear, $P < 0.05$; quadratic, $P > 0.05$) in a comparison with control ($P < 0.05$). Further, the treatments revealed a positive influence on serum cholesterol (linear, $P < 0.05$; quadratic, $P=0.05$) and uric acid ($P > 0.05$) of broiler chickens with respect to control in a dose dependent manner. Other parameters viz. glucose, globuline, triglyceride and hemoglobin were not influenced by EW supplementation.

3.3. Humoral immune response and relative weights of immune organs

The antibody response to both ND and SRBC did not differ among treatments. However, the response to AI increased (linear effect, $P < 0.01$) with dietary EW supplementation (Table 5). The heterophil/lymphocyte ratio, and the relative weight of spleen and thymus did not differ among treatments. On the contrary, increased weight of bursa of Fabricius was observed (linear effect, $P=0.04$) for broiler chickens supplemented with EW compared with control.

3.4. Carcass characteristics and ileum morphology

Dietary treatments had no influence on carcass composition (proportions of liver, thigh, breast, and abdominal fat in body weight) (Table 6). In addition to this, the treatments showed no remarkable variations on the morphology of ileum relative weight, and villus height, length, width, surface, and diameter in a comparison with control.

3.5. Intestinal microbiota

The effect of treatments on the intestinal microbiota is shown in Table 7. The total count of intestinal *E. coli* and other aerobic bacteria were found to be decreased (linear, $P=0.01$) after supplementing various amounts of EW in broiler's diet. On the other hand, broiler chickens fed high dose of EW showed increased counts of (linear, $P=0.01$) intestinal lactic acid bacteria with respect to control ($P=0.01$).

3.6. Meat quality

The treatments at higher doses of EW supplementation revealed increased (linear, $P=0.02$; quadratic, $P=0.01$) MDA of fresh breast, frozen breast (linear, $P=0.01$), and fresh thigh (linear effect, $P=0.01$) in a comparison with control broilers (Table 8). Besides, the water holding capacity of the breast (linear, $P=0.03$; quadratic, $P=0.02$) and thigh (linear, $P=0.04$) was estimated to be decreased as EW was increased.

4. Discussion

4.1. Feed intake, weight gain, and feed conversion

Increasing amounts of EW in the diet of broiler chickens decreased feed intake during the entire experimental period. The reduction in feed intake may be due to the low palatability of the meal for broiler chickens (El Boushy et al., 2000). Besides, Prayogi (2011) concluded that the lack of some essential component in high worm meal diets might be responsible for decreased feed intake due to the decreased appetite at high amounts of inclusion. Fisher (1988) included EW at 72 g/kg of the diet of broiler chickens and observed no effect on the feed intake; however, increasing EW in the diet decreased feed conversion efficiency.

The daily weight gain did not differ between broiler chickens fed EW supplementation or control diet. Sugimura et al. (1984) observed that feeding broiler chickens in diets containing EW had no effect on average weight gain and feed utilization. In addition to this, incorporation of 72 g EW/kg of DM in broiler chickens diet (about 250 g of the dietary protein/kg) did not affect the growth (Fisher, 1988). However, Sofyan et al. (2010) evaluated the effect of EW nutritional supplements

Table 3
Effect of different amounts of earthworm meal (EW) and vermi-humus (VH) on feed intake, weight gain, and feed conversion ratio of broiler chickens.

Item	Treatments[a]					SEM	*P*-value		
	Control (0EW-0VH)	0EW-10VH	10EW-10VH	20EW-10VH	30EW-10VH		Control vs. others	Linear	Quadratic
Feed intake (g/d)									
d0 to 14	33.6	34.8	34.1	33.5	33.6	0.4	0.83	0.79	0.63
d14 to 28	83.4	86.7	85.1	81.7	81.0	0.9	0.29	0.14	0.21
d28 to 42	165.0	163.0	163.9	162.0	155.6	1.0	0.28	0.06	0.46
d 0 to 42	94.1	94.6	95.0	91.7	90.3	0.5	0.01	0.01	0.04
Daily weight gain (g/d)									
d0 to 14	23.5	23.7	23.7	23.5	23.3	0.2	0.98	0.67	0.69
d14 to 28	51.8	54.1	53.7	52.3	52.3	0.6	0.78	0.86	0.33
d28 to 42	83.1	80.9	82.9	85.1	83.9	1.0	0.80	0.45	0.79
d0 to 42	52.8	52.9	53.4	53.6	53.2	0.2	0.65	0.31	0.42
Feed conversion ratio									
d0 to 14	1.42	1.47	1.44	1.42	1.44	0.01	0.78	0.89	0.79
d14 to 28	1.61	1.60	1.58	1.56	1.54	0.01	0.56	0.09	0.86
d28 to 42	1.99	2.00	1.97	1.91	1.86	0.02	0.11	0.01	0.32
d0 to 42	1.78	1.78	1.77	1.71	1.70	0.01	0.02	0.03	0.15

[a] The treatments were: a basal diet without addition of EW or VH (0EW-0VH; control), and the diet containing 10 g VH/kg of DM supplemented with 0, 10, 20, or 30 g EW/kg of DM as a starter diet from d 0 to 14 of the study.

on growth and meat quality of broiler chickens, and observed that feeding broiler chickens with EW increased daily weight gain and improved feed efficiency.

Because of decreased feed consumption without affecting daily weight gain of broiler chickens, feed conversion ratio was improved after the treatments. Prayogi (2011) observed improved feed conversion after feeding EW at 5% and 10% of diet DM. Rezaeipour et al. (2014) also observed that feeding EW at 20–60 g/kg of DM improved the feed to gain ratio of broiler chickens without affecting feed intake and weight gain.

4.2. Blood biochemical measurement

The supplementation of EW increased blood Ca and P concentration. Both Ca and P are essential elements for the structure and metabolism of bone as well as energy accumulation. In agreement with the present investigation, Ozturk et al. (2012) observed that feeding a diet containing humic acid increased plasma Ca and P concentrations.

In the present study, the treatments were responsible for the significant reduction in the cholesterol level as well as reduced glucose concentrations to some extent. Reducing blood cholesterol level shows beneficial effects on the blood lipid profile, thereby indicating positive effect on human health. Feeding humic acid was known to decrease plasma cholesterol concentration (Ozturk et al., 2012). Celik et al. (2008) observed decreased concentrations of cholesterol and glucose after feeding broiler chickens on a diet containing humic acid at 2.5 g/kg of DM.

The incorporation of higher doses of EW increased serum total protein and albumin concentrations. This might be due to the improved proteolytic activity with feeding humic acid. Humic acid feeding is known to increase protein digestion and feed utilization (Islam et al., 2005). The effect of EW on blood metabolites of protein and albumin may be mainly due to the action on proteolytic enzymes as well as precursors of these enzymes.

4.3. Humoral immune response and relative weights of immune organs

The antibodies of AI response were affected as EW supplementation increased, thereby suggesting that EW and humic acid may influence systemic or humoral immunity of broiler chickens. The results revealed the positive influence of EW and humic acid on the response to vaccination of the broiler chicken's immune system. Immune mechanisms of EW include cellular and humoral components. Humoral components of EW include lectins, antimicrobial peptides, pore-forming proteins, phenoloxidases and proteases (Popović et al., 2005). Tohid et al. (2010) observed modified immune function with increase in the antibody of AI in broiler chickens fed diets containing 30 g humic acid/kg of DM due to the ability to form a protective film on the mucous epithelia of the intestine against infections and toxins.

Humic acid has immune-stimulatory, anti-inflammatory, and anti-

Table 4
Effect of different amounts of earthworm meal (EW) and vermi-humus (VH) on blood biochemical measurements of broiler chickens.

Item	Treatments[a]					SEM	*P*-value		
	Control (0EW-0VH)	0EW-10VH	10EW-10VH	20EW-10VH	30EW-10VH		Control vs. others	Linear	Quadratic
Total protein (g/dL)	4.44	4.99	4.82	5.01	5.13	0.09	0.04	0.03	0.50
Albumin (g/dL)	2.52	2.85	2.93	2.92	3.02	0.06	0.04	0.01	0.24
Globulin (g/dL)	2.11	1.92	1.96	2.08	2.06	0.04	0.61	0.86	0.28
Glucose (g/dL)	128.9	100.1	120.9	128.9	127.2	5.1	0.04	0.90	0.14
Cholesterol (g/dL)	160.0	141.2	143.1	142.2	142.1	2.2	0.04	0.03	0.05
Triglyceride (g/dL)	156.2	167.0	148.1	162.0	155.0	6.0	0.91	0.86	0.94
Uric acid (g/dL)	5.93	5.36	5.30	5.48	5.27	0.10	0.18	0.07	0.22
Hemoglobin (g/dL)	12.80	11.81	11.64	12.00	12.04	0.15	0.12	0.17	0.03
Ca (mg/dL)	6.89	8.33	8.31	8.38	8.63	0.21	0.04	0.01	0.15
P (mg/dL)	4.19	5.29	5.18	5.48	5.64	0.19	0.04	0.02	0.32

[a] The treatments were: a basal diet without addition of EW or VH (0EW-0VH; control), and the diet containing 10 g VH/kg of DM supplemented with 0, 10, 20, or 30 g EW/kg of DM as a starter diet from d 0 to 14 of the study.

Table 5
Effect of different amounts of earthworm meal (EW) and vermi-humus (VH) on humoral immune response, heterophil/lymphocyte ratio and relative weights of immune organs of broiler chickens.

Item	Treatments[a]					SEM	*P*-value		
	Control (0EW-0VH)	0EW-10VH	10EW-10VH	20EW-10VH	30EW-10VH		Control vs. others	Linear	Quadratic
Humoral immune response[b]									
SRBC	2.0	2.8	3.4	3.8	3.4	0.3	0.33	0.28	0.21
ND	4.6	6.0	6.6	6.7	7.6	0.5	0.33	0.06	0.83
AI	5.8	6.2	6.4	6.6	6.6	0.1	0.04	0.01	0.35
Heterophil/lymphocyte ratio	0.48	0.37	0.33	0.26	0.29	0.04	0.25	0.05	0.86
Relative weights of immune organs (% body weight)									
Spleen	0.09	0.10	0.13	0.13	0.13	0.01	0.13	0.13	0.03
Thymus	0.19	0.25	0.25	0.25	0.29	0.01	0.01	0.01	0.36
Bursa of Fabricius	0.08	0.11	0.13	0.13	0.15	0.01	0.04	0.04	0.59

[a] The treatments were: a basal diet without addition of EW or VH (0EW-0VH; control), and the diet containing 10 g VH/kg of DM supplemented with 0, 10, 20, or 30 g EW/kg of DM) as a starter diet from d 0 to 14 of the study.
[b] SRBC- Sheep red blood cell; ND- Newcastle disease; AI- Avian Influenza

Table 6
Effect of different amounts of earthworm meal (EW) and vermi-humus (VH) on carcass characteristics, and ileum morphology and characteristics of broiler chickens.

Item	Treatments[a]					SEM	*P*-value		
	Control (0EW-0VH)	0EW-10VH	10EW-10VH	20EW-10VH	30EW-10VH		Control vs. others	Linear	Quadratic
Carcass (g/kg of body weight)									
Liver	19.5	23.4	23.9	21.2	23.3	0.8	0.11	0.13	0.13
Thigh	253.0	246.0	24.4	250	249.2	2.1	0.65	0.73	0.27
Breast	264.0	272.0	271.0	289.0	284.0	4.0	0.19	0.24	0.16
Abdominal fat	22.4	231.0	21.5	15.5	18.4	1.4	0.40	0.51	0.20
Ileal/Ileum morphology									
Villus height (μm)	742.0	754.0	777.1	816	834.0	17	0.50	0.05	0.84
Villus diameter (μm)	83.3	84.8	79.3	79.3	79.8	3.0	0.97	0.58	0.89
Villus depth (μm)	38.1	38.3	38.7	38.7	35.6	0.91	0.99	0.86	0.89
Villus surface (μm^2)	30.78	32.4	30.5	32.2	33.0	1.25	0.97	0.66	0.87
Villus height/villus depth	19.6	19.9	20.5	21.3	21.7	0.6	0.76	0.19	0.97
Relative weight (g/kg)	15.2	17.8	17.7	17.6	18.7	0.7	6.3	0.27	0.31
Length (cm)	66.0	68.0	70.2	75.8	77.0	1.6	0.11	0.01	0.88
Width (mm)	6.15	6.81	7.72	8.12	8.51	0.34	0.02	0.12	0.69
Diameter (mm)	0.27	0.32	0.37	0.40	0.45	0.10	0.03	0.30	0.97

[a] The treatments were: a basal diet without addition of EW or VH (0EW-0VH; control), and the diet containing 10 g VH/kg of DM supplemented with 0, 10, 20, or 30 g EW/kg of DM) as a starter diet from d 0 to 14 of the study.

viral properties (Klocking, 1994). Some possible modes of action include phagocytic activity of leukocytes, adhesibility, and activation of neutrophils (Chong-Hua et al., 2003), thereby improving nutritive value of feed in order to block colonization of pathogens in the gastrointestinal tract (Klocking, 1994), and improving immune functions (Eren et al., 2000). The ingestion of humic acid increases the *Lactobacillus* and *Bifidobacterium* colonization, causing modulated immune system. In spite of the ability of *Bifidobacterium* to stimulate the antibody production, it is well proved that *Lactobacillus* is an immunomodulator due to its ability to reduce the bacterial translocation in animals (Bornet et al., 2002). Moreover, cytolisin of EW coelomic fluid (Eiseniapore) was reported to cause pore like structures at the target membranes (Lange et al., 1999). Cooper et al. (2002) showed that specific molecules in the earthworm's immune system might be used as a natural antibiotic. Earthworm meal supplementation increased the bursa of Fabricius weight without affecting the heterophil/lymphocyte ratio. Further, the relative weight of spleen and thymus reveal great impact on humoral immune system of the broiler chickens.

4.4. Carcass characteristics and ileum morphology

Inclusion of various amounts of EW in the diet of the broiler chickens had no effect on internal organs weights and the morphology of the ileum. To the best of our knowledge, there are very few reports

Table 7
Effect of different amounts of earthworm meal (EW) and vermi-humus (VH) on intestinal microbial communities (log cfu/g of intestinal contents) of broiler chickens.

Item	Treatments[a]					SEM	*P*-value		
	Control (0EW-0VH)	0EW-10VH	10EW-10VH	20EW-10VH	30EW-10VH		Control vs. others	Linear	Quadratic
E. coli	5.06	3.56	5.24	3.74	3.75	0.23	0.01	0.01	0.30
Lactic acid bacteria	6.25	6.27	6.57	6.74	6.78	0.09	0.01	0.01	0.33
Total count of aerobic bacteria	6.46	5.95	5.74	5.29	5.35	0.12	0.01	0.01	0.69
Aerobic spore bacteria	4.31	3.99	3.90	3.84	3.93	0.05	0.01	0.01	0.33

[a] The treatments were: a basal diet without addition of EW or VH (0EW-0VH; control), and the diet containing 10 g VH/kg of DM supplemented with 0, 10, 20, or 30 g EW/kg of DM as a starter diet from d 0 to 14 of the study.

Table 8
Effect of different amounts of earthworm meal (EW) and vermi-humus (VH) on meat quality of broiler chickens.

Item	Treatments[a]					SEM	*P*-value		
	Control (0EW-0VH)	0EW-10VH	10EW-10VH	20EW-10VH	30EW-10VH		Control vs. others	Linear	Quadratic
Malondialdehyde (μg/g)									
Fresh breast	0.18	0.16	0.20	0.30	0.36	0.02	0.02	0.02	0.01
Frozen breast	0.44	0.44	0.53	0.56	0.83	0.04	0.01	0.01	0.09
Fresh Thigh	0.45	0.41	0.32	0.58	1.06	0.07	0.01	0.01	0.12
Frozen tight	0.81	0.50	0.83	0.87	0.96	0.05	0.01	0.01	0. 49
Water holding capacity									
Breast	0.59	0.63	0.60	0.58	0.56	0.01	0.03	0.03	0.02
Tight	0.67	0.68	0.66	0.63	0.62	0.01	0.04	0.04	0.75
pH									
Breast	6.30	6.33	6.27	6.22	6.20	0.01	0.01	0.01	0.01
Tight	5.95	6.12	5.84	5.83	5.78	0.03	0.01	0.01	0.01

[a] The treatments were: a basal diet without addition of EW or VH (0EW-0VH; control), and the diet containing 10 g VH/kg of DM supplemented with 0, 10, 20, or 30 g EW/kg of DM as a starter diet from d 0 to 14 of the study.

on the effect of feeding EW and VH on gastrointestinal morphology of broiler chickens. Generally, the feed dietary nutrients utilization partially depends on the development of the gastrointestinal tract. Measuring villus height, diameter, depth, and surface area are indicators about the area available for digestion and absorption, and the activities of membrane-bound digestive enzymes of the small intestine. Unaffected intestinal villi reveal normal nutrient absorption from digestive tract towards blood. In agreement with the current results, Sugimura et al. (1984) observed that feeding broiler chicken on diets containing EW did not affect liver, kidney, muscle, spleen, and pancreas weights. However, Rezaeipour et al. (2014) observed that due to the presence of Gln in the meal, feeding EW at 40 g/kg of DM of diet caused bigger jejunum crypt depth in a comparison with 20 g EW/kg of DM. In the present study, the dose dependent supplementation of EW into the diet caused significant increment in the villus height in a comparison with the control treatment. However, this effect was not observed on the ileum villi morphology. Soltan (2009) observed that feeding broiler chickens on diet containing Gln increased villi height in duodenum and jejunum.

4.5. Intestinal microbiota

Gastrointestinal microbiota is one of the factors responsible for the health of poultry. In the present context, feeding EW caused a shift on the intestinal microbial profile in terms of decreased intestinal *E. coli* and total count of aerobic bacteria. Further, the supplementation of EW at varied doses increased intestinal lactic acid bacteria. Loh et al. (2009) observed that the total count of lactic acid bacteria was increased with feeding EW to broiler chickens. Popović et al. (2005) observed that EW constitutes glycolipoprotein (G-90) and a mixture of homogeneous tissue, conferring potent antibacterial activity against *Staphylococcus* sp., higher than that of antimicrobials such as gentamicin and enrofloxacin. In addition to this, EW was reported to constitute OEP3121 peptides with antimicrobial activity (Liu et al., 2004). Feeding humic acid has the unique potentiality in terms of antidiarrheal, analgesic, immunostimulatory and antimicrobial characteristics. Chong-Hua et al. (2003) showed that the immune response of animals could be mediated by macrophages activation because of the ability of humic acid to activate macrophages. Improving immune functions can reduce the incidence of diarrhea and other digestive upsets. Celik et al. (2008) showed that pathogenic microbes can affect broiler chickens growth as it has the potentiality to produce toxins, thereby reducing the utilization of nutrients essential to the host animal and suppressing the microbes responsible for the synthesize of vitamins and other host growth factors. Ozturk et al. (2012) observed that total counts of aerobic mesophilic bacteria, lactic acid bacteria, and *E. coli* were decreased after feeding humic acid at 25.5 mg/kg of body weight. Such results revealed that the EW supplementation could be used as a safe and organic alternative for antibiotic and ionophores due to the potential appearance of residues since their usage has been banned in the European Union (Islam et al., 2005).

4.6. Meat quality

Determination of TBARS is an efficient way to measure antioxidant activity in meat products. Analyzing TBARS concentration is an indicator of MDA, a product of oxidation as the values of TBARS increase with increasing storage period. Malondialdehyde is the main product of the peroxidation of polyunsaturated fatty acids, and can be used as an indicator of lipid peroxidation (Freeman and Crapo, 1981). The response of MDA value with feeding EW differed in terms of increased MDA of fresh and frozen breast. Decreased MDA value revealed that the antioxidant activity in meat increased during storage with EW supplementation. To the best of our knowledge, this is the first report evaluating the meat quality of broiler chickens fed varied amounts of EW.

Meat quality can be altered through changing feeding strategies. The roles of dietary protein have gained an immense interest in influencing growth and body composition of livestock; regulating the expression of key lipogenic genes, and the growth of white adipose tissue (Libién-Jiménez et al., 2015; Liu et al., 2015). Moreover, maintaining the dietary fatty acids would facilitate the absorption and utilization of fatty acids and free amino acids, and would result in improved muscle and adipose composition (Li et al., 2015). Meat quality can be used as an indicator of stress and energy metabolism in poultry (Ozturk et al., 2012). Meat quality can be measured by determining meat color, pH, and water-holding capacity (WHC) as they can affect consumer preferences. In the present study, feeding broiler chickens with higher dose of EW caused measurable changes in pH of breast, thigh meats, and WHC, which affects positively on consumer acceptance (Ozturk et al., 2012). Meat pH is a direct reflection of muscle acid concentration, affecting meat drip loss and color. Ozturk et al. (2012) observed that the addition of humic acid to the diets of broiler chickens had no influence on thigh muscle pH.

Without affecting the WHC value in breast meat, feeding EW decreased thigh WHC which may encourage liquid outflow, and loss of soluble nutrients and flavor, thereby causing some negative impact on meat quality as it becomes dry, hard and tasteless. A difference between the WHC of thigh and breast muscles may be due to the difference in muscle fiber type, and also due to the fact that breast muscle is more sensitive than thigh muscle (Lonergan et al., 2003).

5. Conclusion

Feeding various concentrations of EW did not affect weight gain but decreased feed intake consequently, thereby improving feed efficiency. Higher blood protein Ca and P concentrations, better humoral immune response, and increased immune organs are other benefits of EW supplementation. The intestinal microbial profile was improved with feeding dietary EW. Meat quality improvement was undoubtedly dependent on the supplementation of variable concentrations of EW. The diet containing higher doses of EW supplementation showed paramount importance to broiler chickens.

Conflict of interest

None declared.

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