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Tenebrio meal as a functional ingredient modulates immune response and improves growth performance of broiler chickens

J. M. M. Andrade [*,1] **R. T. Pereira** [*] **V. R. C. de Paula** [†] **H. Moreira Junior** [*] **and J. F. M. Menten**[*]

[*]*Department of Animal Science, University of São Paulo - ESALQ, Piracicaba, São Paulo 13418-900, Brazil; and* [†]*Institute of Agrarian and Environmental Sciences- ICAA, Federal University of Mato Grossso - UFMT, Sinop, Mato Grosso 78550-728, Brazil*

Primary Audience: Poultry Nutritionists, Poultry Industry, Poultry Production, Researchers

SUMMARY

Two experiments were carried out to evaluate the use of full-fat tenebrio larvae meal (**TM**) in broilers diet. In experiment 1, 800 birds were assigned to 4 treatments in an open-sided poultry house, during 35 d, to evaluate performance and count of selected bacteria in ceca. Dietary treatments were as follows: negative control (**NC**); positive control (**PC**)—NC + 10 mg/kg enramycin and 66 mg/kg salinomycin; **TM0.5**—NC + 0.5% TM; and **TM2.0**—NC + 2.0% TM. In experiment 2, the same treatments were used; 192 birds were raised in battery cages for 21 d. At the end of the trial, birds were challenged with LPS and blood was sampled for innate immune response evaluation. PROC MIXED of SAS was used in statistical analyses and comparisons of means done using Tukey's test. In experiment 1, treatment TM2.0 resulted in increase in BWG and FI of 3.8 and 4.2% ($P < 0.05$), and in experiment 2 the improvements were of 5.7 and 2.1% ($P < 0.05$), compared to NC. TM2.0 improved FCR relative to NC in experiment 2 ($P < 0.05$), but not in experiment 1. In both trials the supplementation with 0.5% TM did not improve performance. Birds nonchallenged with LPS (**NCLPS**) and fed 2.0% TM had hemolytic activity of the alternative complement system (**HACS**) and myeloperoxidase activity (**MPO**) increased compared to NC and even PC ($P < 0.05$). The indication of TM as a functional ingredient is ascertained by the growth improvement and innate immune response modulation in chickens fed the diet containing 2.0% TM.

Key words: antibiotic growth promoter, feed additive, ionophore, poultry, *Tenebrio molitor*

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DESCRIPTION OF PROBLEM

The use of insect larvae meal as an ingredient in broilers feed has been gaining attention for food-producing animals. In recent years, there has been an increase in published research on insect as feed, but studies to define its application in poultry nutritional programs, such as inclusion levels, are still necessary. Moreover, insect meal could play a role beyond the nutritional requirements by being a functional ingredient, which could be defined in virtue of the presence of physiologically active components,

[1]Corresponding author: josematheus@usp.br

providing a health benefit beyond basic nutrition (Hasler, 2002). Nutraceutical is a comprehensive umbrella term that is used to define the biologically active components of food which provide the extra health benefits in addition to the basic nutritional value (Hasler, 2002).

Tenebrio molitor, a species of the order Coleoptera, family Tenebrionidae, is a beetle whose larval form is called mealworm. Its larvae meal has 44 to 69% crude protein and 23 to 47% fat on dry matter basis (Veldkamp et al., 2012; Makkar et al., 2014). The protein has a good amino acid profile, comparable to soybean or fish meal protein (De Marco et al., 2015).

Insects such as tenebrio and others are able to synthesize antimicrobial peptides (**AMP**) as a protective mechanism (Bulet et al., 2004; Józefiak and Engberg, 2017). AMP are proteins with short amino acid sequence (<100) and have shown microbicidal effect against bacteria, virus, fungi, and other pathogens (Jenssen et al., 2006; Mylonakis et al., 2016; Józefiak and Engberg, 2017). Four AMP are described for tenebrio, *tenecin 1, 2, 3 and 4*, which are active against gram-negative and positive bacteria and against fungi (Moon et al., 1994; Lee et al., 1996; Roh et al., 2009; Chae et al., 2012). Insects are also rich in chitin and chitosan which possess microbicidal activity (Vartiainer et al., 2004) and can initiate an immune response and modulation after recognition by *toll-like receptors* (Lee et al., 2008; Islam and Yang, 2017; Komi et al., 2018). According to Fuchs et al. (2018), *toll-like receptor* 2 is a mammalian immune cell pattern recognition receptor that directly binds chitin with high affinity. In this way, after the recognition of the immune system, immune responses can be triggered.

Most nutritional studies with insect meals have focused on their nutritive value as a high protein and lipid animal ingredient (Veldkamp et al., 2021); however, considering the costs and availability, evaluating the effects of insect meal as a functional ingredient at low levels is an attractive research topic. In a previous study conducted by our research group, diets formulated with 0, 4, 8, or 12% of full-fat tenebrio larvae meal (**TM**) were fed to broilers raised in floor pens from 1 to 35 d. Feed containing 4% TM resulted in significant increase of 140 g in body weight gain (**BWG**) with modulatory effect of the innate immune response on broilers challenged with lipopolysaccharide (**LPS**) (unpublished). Furthermore, Benzertiha et al. (2020) demonstrated that the inclusion of small amounts (0.2 or 0.3%) of TM in broiler diets improved growth performance and modulated the immune response, as indicated by reduced serum IgM and increased serum IL-2 and TNF-α levels. In this case, improvement in growth performance cannot be attributed to the nutrient contribution of the insect meals, and some nutraceutical properties might be involved.

Therefore, we hypothesized that low inclusion levels of 0.5 or 2.0% of TM in broiler diets may improve growth performance and modulate the innate immune response of broiler chickens. The effects of TM on growth performance and serum innate immunity in broilers challenged with LPS from *Escherichia coli* were also investigated. To the best of our knowledge this is the first study to evaluate the effects of TM on the innate immune response of broiler chickens under an LPS-induced inflammation.

MATERIALS AND METHODS

This study was conducted at Department of Animal Science, University of São Paulo (Piracicaba, SP, Brazil). All procedures were approved by the Institutional Animal Care and Use of Committee (protocol number: 8400081020).

Two experiments were carried out with the use of TM in the feed for broiler chickens. The first feeding trial assessed broiler chickens growth performance and bacterial counts in the intestine and the second trial evaluated the innate immune response under an LPS-induced inflammation.

In both feeding trials, diets met the nutritional requirements of the Brazilian Tables for Poultry and Swine (Rostagno et al., 2017), based on corn and soybean meal, being isonutritive and isoenergetic. Diets were prepared in mash form. In experiment 1, starter diets were offered from 1 to 21 d of age and grower diets from 22 to 35 d of age; in experiment 2, only

Table 1. Analyzed nutrient composition of the *Tenebrio molitor* full-fat larvae meal (TM) used in the experiments, as fed basis.

Item	*Tenebrio molitor*
Dry matter	97.02
Crude protein	50.00
Ether extract	29.73
Crude ash	4.12
Crude fiber	4.50
Calcium	0.125
Phosphorus	0.570
Gross energy (kcal/kg)	6366
AMEn (kcal/kg)	4854
Indispensable amino acids	
Arginine	2.68
Histidine	1.48
Isoleucine	1.91
Leucine	3.31
Lysine	2.90
Methionine	0.68
Phenylalanine	2.12
Threonine	1.71
Valine	2.76
Dispensable amino acids	
Alanine	3.16
Aspartic acid	3.89
Cysteine	0.54
Glycine	2.25
Glutamic acid	5.58
Proline	2.85
Serine	1.72
Tyrosine	4.07

Values in % unless otherwise stated.

the starter diet was utilized. The experimental treatments were as follows: Negative control (**NC**)—no additives; Positive control (**PC**)—NC + 10 mg/kg enramycin and 66 mg/kg salinomycin; **TM0.5**—NC + 0.5% TM; and **TM2.0**—NC + 2.0% TM. TM was analyzed for dry matter, crude protein, ether extract, ash, crude fiber, gross energy, calcium, phosphorus, and amino acid profile. We have previously determined that the standardized ileal digestibility coefficient of amino acids in TM is greater than 0.81 (Nascimento Filho et al., 2021). The methodology of bromatological analysis can be found in the publication of Nascimento Filho et al. (2021). TM was obtained from Vida Proteína Cia. Ltda., Neirópolis, Goiás, Brazil and its analyzed composition is depicted in Table 1. Diet composition is shown in Table 2.

Randomized complete block design was used in both experiments. We adopted this design because the poultry house used for experiment 1 has trees on only one side and an even distribution of treatments was preferred.

Experiment 1

Animals and Experimental Procedures. Experiment 1 was set to evaluate growth performance. Chickens were raised in an open-sided poultry house equipped with curtains, fans, foggers, heating lamps, nipple drinkers, and tube feeders, in 3.0 m^2 floor pens covered with reused rice hulls litter. Environmental conditions were maintained close to the chickens needs at each stage of growth.

A total of 800 one-day-old male broiler chicks (Cobb 500) were obtained from a local hatchery, weighed individually upon arrival, uniformly distributed to groups and randomly assigned to 40 pens. Chicks, with average weight of 45.3 g, were divided into 4 treatments with 10 replicates (20 birds/pen).

At the age of 7, 14, 21, 28, and 35 d, the birds were weighed on a pen basis and feed consumption was recorded. Weekly data and cumulative values for BWG and feed intake (**FI**) were calculated, and feed conversion ratio (**FCR**) was calculated, adjusted for mortality.

At the end of the trial (35 d of age), 8 birds per treatment, randomly taken from 8 pens in each treatment, were sacrificed and the ceca was quickly removed. The left ceca were collected and sent to a private laboratory for bacterial counting analysis of *Escherichia coli* and *Enterococcus* spp. according to APHA (2001) methodology (MKBLAB, Jundiaí, São Paulo, Brazil).

Experiment 2

Animals and Experimental Procedures. Experiment 2 was designed to evaluate bird immune response, conducted in a ventilated poultry house, in battery cages with heating control, wire mesh floors and stainless-steel trough feeders and waterers. During the entire experiments, chickens had ad libitum access to water and feed.

A total of 192 one-day-old male broiler chicks (Cobb 500) were obtained from a local hatchery, weighed individually upon arrival, and randomly divided into 24 metallic cages.

Table 2. Ingredient and nutrient composition of the experimental diets[1] (experiments 1 and 2).

	Treatments							
	NC	PC	TM0.5	TM2.0	NC	PC	TM0.5	TM2.0
Items	Starter (1−21 d)				Grower (21−35 d)			
Ingredients (g/kg)								
Corn	490.3	489.5	494.0	505.1	564.0	563.2	654.6	587.1
Soybean meal	434.0	434.1	427.8	409.4	353.9	354.0	353.1	322.9
Soybean oil	36.8	37.0	34.2	26.4	49.2	49.5	48.9	37.3
Dicalcium phosphate	18.0	18.0	18.1	18.2	14.7	14.7	14.7	15.0
Limestone	8.9	8.9	8.9	8.9	7.0	7.0	7.0	7.0
Salt	5.3	5.2	5.2	5.2	4.9	4.9	4.9	4.9
Mineral premix[a]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin premix	0.5 [b]	0.5	0.5	0.5	0.4 [c]	0.4	0.4	0.4
DL-Methionine	3.239	3.239	3.236	3.230	2.740	2.741	2.741	2.791
L-Lysine HCl 77%	1.221	1.218	1.226	1.242	1.523	1.521	1.529	1.764
L-Threonine	0.483	0.483	0.481	0.475	0.408	0.408	0.410	0.496
Choline chloride	0.8	0.8	0.8	0.8	0.6	0.6	0.6	0.6
Tenebrio molitor full-fat meal	0	0	5.0	20.0	0	0	0.5	20.0
Enramycin 8%	0	0.125	0	0	0	0.125	0	0
Salinomycin 24%	0	0.275	0	0	0	0.275	0	0
Calculated values (g/kg)								
Crude protein	239.00	239.00	239.00	239.00	208.38	208.37	208.32	206.02
Available phosphorus	4.45	4.45	4.45	4.45	3.74	3.74	3.74	3.74
Calcium	9.35	9.35	9.35	9.35	7.58	7.58	7.58	7.58
Sodium	2.22	2.22	2.22	2.22	2.08	2.08	2.08	2.08
Dig. methionine	6.34	6.34	6.34	7.58	5.51	5.51	5.51	5.52
Dig. lysine	12.90	12.90	12.90	12.90	11.24	11.24	11.24	11.24
Dig. methionine + cysteine	9.50	9.50	9.50	9.50	8.32	8.32	8.32	8.32
Dig. threonine	8.50	8.50	8.50	8.50	7.42	7.42	7.42	7.42
AME (kcal/kg)	2975	2975	2975	2975	3150	3150	3150	3150

[a]Salus mineral products, provided the following per kilogram of diet: manganese, 80 mg; zinc, 70 mg; iron, 50 mg; copper, 10 mg, and iodine, 1 mg.

[b]Salus vitamin products, provided the following per kilogram of diet: vitamin A, 8,500 IU; vitamin D_3, 3,000 IU; vitamin E, 18 IU; vitamin K_3, 2,5 mg; Vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_6, 3 mg; vitamin B_{12}, 14 μg; vitamin B_5, 14 mg; folic acid, 1.2 mg; biotin, 0.008 mg, and selenium, 0.5 mg.

[c]Salus vitamin products, provided the following per kilogram of diet: vitamin A, 6,800 IU; vitamin D3, 2,400 IU; vitamin E, 14 IU; vitamin K_3, 2,0 mg; vitamin B_1, 1.6 mg; vitamin B_2, 4.8 g; vitamin B_6, 2.4 mg; vitamin B_{12}, 11 μg; vitamin B_5, 11 mg; folic acid, 1.0 mg; biotin, 0.006 mg, and selenium, 0.4 mg.

[1]NC = negative control; PC = positive control—NC + 10 mg/kg enramycin + 66 mg/kg salinomycin; TM0.5—NC + 0.5% *Tenebrio molitor* full-fat meal; TM2.0—NC + 2.0% *Tenebrio molitor* full-fat meal. All diets had no phytase.

Chicks, with 42.5 g initial BW, were divided into 4 treatments, with 6 replicates (8 birds/cage). On 7, 14, and 21 d of the experiment, the birds were weighed on a cage basis and feed consumption was recorded. Weekly data and cumulative values for BWG and FI were calculated, and FCR was calculated, adjusted for mortality.

At 21 d of age, 1 bird/pen (n = 6) was inoculated i.p. with 2 mL (1.48 mg/kg of BW) *Escherichia coli* LPS (serotype O55:B5, LPS L2880; Sigma-Aldrich) and 1 bird/pen (n = 6) of the same pen was inoculated i.p. with 2 mL of 0.9% (w/v) sterile saline solution as negative control. After 6 h, blood was collected from the brachial vein into 4 mL clot accelerator tubes to obtain serum for the analysis of the innate immune system. Then, blood samples were centrifuged at 2,000 × g for 10 min at 4˚C, aliquoted in Eppendorf vials and stored at −80˚C.

After serum collection, analyses were performed to assess the response of the innate immune system: lysozyme activity (**LYZ**), hemolytic activity of the complement system (**HACS**), myeloperoxidase activity (**MPO**) and bactericidal activity (**BA**) against *E. coli* and BA against *Salmonella* Gallinarum. All these analyses were carried out in serum of the birds challenged with LPS (**CLPS**) and nonchallenged birds (**NCLPS**).

Measurements and Analytical Methods

Lysozyme Activity. The LYZ of birds was determined by a turbidimetric assay as described by Jorgensen et al. (1993). Briefly, a suspension (200 μL) of Micrococcus lysodeikticus (M3770, Sigma-Aldrich) in PBS (0.2 g/L) at pH 6.2 was mixed with serum (10 μL) in a flat-bottomed 96-well plate. Plates were read with a microplate reader (Synergy H1 Multi-Mode Reader, Winooski, VT). Lysozyme activity (units/mL) was calculated using the following formula: [(Δabsorbance (4 min/min)/3)/0.001] × 100. Quantification of lysozyme activity was done as per the standard definition of 1 unit of lysozyme activity from chicken egg hen lysozyme (L6876, Sigma Aldrich) corresponding to the linear decrease in optical density (**OD**) at 450 nm of 0.001 per min.

Hemolytic Activity of the Alternative Complement System. The HACS was measured as described by Sutili et al. (2016). An 80% diluted sample (80 μL of serum + 20 μL PBS) was mixed with 100 μL of 2% washed sheep red blood cells (**SRBC**) and incubated at 25°C for 45 min. Following incubation, the mixture was centrifuged at 2,500 × *g* for 5 min. Then, 100 μL of supernatant were transferred to a 96-well plate and measured at 450 nm in a plate reader (Synergy H1 Multi-Mode Reader, Winooski, VT). The percent of hemolysis was calculated by comparing between total hemolysis (100%: SRBCs + DI water) and no-hemolysis (0%: SRBCs + PBS + heat-inactivated serum) controls as follows: % hemolysis = [(A_{450} sample − A_{450} no-hemolysis)/(A_{450} total hemolysis − A_{450} no-hemolysis)] × 100.

Myeloperoxidase Activity. The MPO in serum was quantified following the protocol described by Kreutz et al. (2011), with modifications. Ten microliters of serum were diluted in 40 μL of PBS in flat-bottomed 96-well plates. Then, 100 μL of a solution containing o-3,3′, 5,5′-tetramethylbenzidine (**TMB**, T0440, Sigma-Aldrich) and hydrogen peroxide prepared in citrate (0.2 M) and a phosphate buffer (0.01 M) at pH 5.3 was added to each well. The peroxidase reaction was stopped after 5 min by adding 100 μL of HCl (3 M). Plates were read with a microplate reader (Synergy H1 Multi-Mode Reader, Winooski, VT), and the myeloperoxidase activity reported in absorbance at 450 nm. Wells without samples were added in the microplate as negative controls.

Bactericidal Activity. The serum BA was determined by evaluating its effect on growth of *Salmonella* Gallinarum and *E. coli* bacteria. The bacterial strains were kindly provided by Dr. Leticia Gressler (Laboratory of Veterinary Microbiology and Immunology, Farroupilha Federal Institute of Education, Science and Technology). The bacterial solution for each strain was prepared in tryptone soy broth medium (**TSB**) from cultures grown on tryptone soy agar medium (Himedia Laboratories) {(1 × 10^8 colony forming units (**CFU**)/mL; 0.15 optical density at 600 nm) (30°C/24 h)}. Then, 20 μL of serum and 20 μL of bacterial dilution at a concentration of 10^6 bacteria/mL were added to each well of 96-well plates. The samples were incubated for 4h at 37°C. After incubation, 25 μL/well of 2,3,5-triphenyltetrazolium chloride, 0.5 mg/mL (Sigma) were added and the samples were incubated for another 10 min (37°C), before being centrifuged (2,000 × *g*, 10 min). The supernatant was removed and the precipitate dissolved with 200 μL/well of dimethylsulfoxide. Aliquots of 100 μL from each well were transferred to a new plate of 96 flat-bottomed wells. Absorbance was then measured at 450 nm (Synergy H1 Multi-Mode Reader, Winooski, VT). The percent of BA was calculated by comparing between controls: 0% BA (TSB + bacterial solution) and 100% BA (PBS) (Albaladejo-Riad et al., 2020).

All these analysis (LYZ, HACS, MPO, BA *E. coli*, and BA *Salmonella* Gallinarum) were carried out in serum of the birds challenged with LPS (**CLPS**) and nonchallenged birds (**NCLPS**).

Statistical Analysis

Growth performance and bacterial count data were submitted to PROC MIXED (Linear Mixed Models) of SAS software. All data were tested for normality of residuals through Shapiro-Wilk test. When a significant effect was verified, the variables were submitted to mean comparison by Tukey test considering the level of 5% of significance. Serum immune parameters (LYZ, HACS, MPO, and BA) were subjected to Tukey-Kramer test to compare estimated means among treatment CLPS and NCLPS groups separately. Results were reported as least square means with standard error of the mean (**SEM**).

RESULTS AND DISCUSSION

Performance

In experiment 1, broiler chickens fed 2% of TM had improved growth performance

Table 3. Growth performance of broiler chickens fed diets containing antimicrobial additives or 0.5 or 2% of full-fat tenebrio larvae meal (experiment 1, 1−35 d).

	Treatments[1]					
Item[2]	NC	PC	TM0.5	TM2.0	SEM[3]	*P* value
1−7 d						
BWG, g	161[b]	168[a]	163[ab]	168[a]	1.86	0.010
FI, g	169	175	174	175	2.14	0.161
FCR, g:g	1.052	1.037	1.066	1.043	0.013	0.208
7−14 d						
BWG, g	320[c]	383[a]	328[b,c]	364[ab]	11.11	<0.001
FI, g	414[c]	461[a]	420[bc]	452[ab]	10.33	0.005
FCR, g:g	1.298[c]	1.206[a]	1.290[bc]	1.253[ab]	0.015	<0.001
14−21 d						
BWG, g	566[b]	636[a]	561[b]	591[b]	9.48	<0.001
FI, g	731[b]	784[a]	727[b]	763[ab]	10.08	<0.001
FCR, g:g	1.292[b]	1.233[a]	1.295[b]	1.291[b]	0.010	<0.001
1−21 d						
BWG, g	1047[b]	1187[a]	1052[b]	1123[a]	18.72	<0.001
FI, g	1314[c]	1420[a]	1321[bc]	1390[ab]	19.70	0.008
FCR, g:g	1.256[b]	1.196[a]	1.256[b]	1.239[b]	0.007	<0.001
21−28 d						
BWG, g	750[b]	786[a]	746[b]	759[ab]	9.58	0.015
FI, g	1092[b]	1135[a]	1088[b]	1136[a]	10.18	<0.001
FCR, g:g	1.458	1.446	1.460	1.498	0.014	0.055
28−35 d						
BWG, g	910	918	920	927	11.18	0.683
FI, g	1392[b]	1415[a]	1404[ab]	1432[a]	10.72	0.022
FCR, g:g	1.531	1.544	1.526	1.548	0.014	0.640
21−35 d						
BWG, g	1659	1703	1666	1685	15.68	0.135
FI, g	2484[c]	2550[ab]	2492[bc]	2568[a]	16.45	<0.001
FCR, g:g	1.498	1.498	1.496	1.525	0.012	0.272
1−35 d						
BW, g	2752[c]	2936[a]	2764[c]	2853[b]	19.81	<0.001
BWG, g	2706[c]	2890[a]	2718[c]	2808[b]	22.95	<0.001
FI, g	3798[b]	3970[a]	3813[b]	3958[a]	30.53	<0.001
FCR, g:g	1.403[b]	1.374[a]	1.403[b]	1.410[b]	0.006	0.002

Chicks initial BW in g: 45.4; 45.4; 45.2; 45.2, respectively; $P = 0.504$.

[1]NC = negative control; PC = NC + 10 mg/kg enramycin + 66 mg/kg salinomycin; TM0.5 = NC + 0.5% *Tenebrio molitor* full-fat meals and TM2.0 = NC + 2.0% *Tenebrio molitor* full-fat meals.

[2]BWG = body weight gain; FI = feed intake; FCR = feed conversion ratio (g of FI/g of BWG, g/g).

[3]SEM: standard error of the mean.

[a,b,c]Means values within a row having different superscripts are statistically different by Tukey's test ($P < 0.05$).

compared to NC group (Table 3). BWG was significantly higher for the chickens fed 2% TM on wk 1 (+7.1 g, $P < 0.05$), wk 2 (+44 g, $P < 0.05$) and wk 4 (+9.0 g, $P < 0.05$). Although the advantage of TM2.0 compared to NC was not significant ($P > 0.05$) in the remaining weeks, numerical differences were observed, resulting in higher BWG in the starter period, 1 to 21 d (+76 g, $P < 0.05$) and a higher final BWG at 35 d (2,808 g vs. 2,706 g, $P < 0.05$). This represents 7.2% increase in the BWG in the starter phase and 3.8% increase in the total period for the broilers fed TM2.0 over the controls. However, in experiment 2, broiler chickens fed diet containing 2% TM had higher BWG than the NC on wk 3 (+53 g, $P < 0.05$), but there was no significant difference between these treatments at d 21 (1,113 g vs. 1,053 g, $P > 0.05$), as shown in Table 4.

In the first trial, broilers fed the diet supplemented with enramycin + salinomycin had a greater response in BWG in relation to the NC than TM2.0. The response in BWG was 13.4% in the starter phase (1,187 g vs. 1,047 g, $P < 0.05$) and 6.7% in the overall period (2,890 g vs. 2,706 g, $P < 0.05$). PC broilers had higher BWG than TM2.0 at the end of the trial (+82 g, 2.9% higher, $P < 0.05$). In both trials, there was no response in growth performance of broilers receiving the diet TM0.5 in any phase of the trials (Tables 3 and 5).

TM as a functional ingredient may be regarded as a substitute for antimicrobials in feed. Results from a recent meta-analysis (Cardinal et al., 2019) indicated that the response AGPs in BWG was similar to that obtained with TM2.0 in this study (3.8%). In the present case, however, the growth response to TM was 55% of that of that obtained with a combination of enramycin and salinomycin.

In experiment 1, the improvement in BWG of the chickens fed TM2.0 diet was evident on the first week and it continued for the entire growth period of 35 d. At 21 d of age, the difference in BWG was 76 g; in experiment 2, which was terminated at 21 d, the difference in BWG at this age was 60 g. In other studies, broilers fed TM had better BWG only in the starter phase. This was reported by Biasato et al. (2017), with diets containing 0, 5, 10, or 15% TM, and Sedgh-Gooya et al. (2021), feeding a 2.5% TM diet; the authors indicated that this was due to a better efficiency of utilization of nutrients in the initial period. In a previous study, Ballitoc and Sun (2013) also obtained an increase in broiler BWG when fed 2.0% TM, but not 0.5%.

A series of studies have been published recently using elevated inclusion levels of TM in the diet of broiler chickens, considering its nutritional value as an alternative protein ingredient. The insect meal was used in partial or total substitution to soybean meal (Bovera et al., 2016; Biasato et al., 2017; Elahi et al., 2020) or fish meal (Zadeh et al., 2020). A different approach, using insect meal as a functional ingredient has been addressed by the current study.

Despite the failed attempt in our feeding trial to show efficacy of insect inclusion as low as 0.5% of TM, a recent report of Benzertiha et al. (2020) was successful in obtaining improved growth performance of broilers receiving diets containing levels as low as 0.2 or 0.3% TM in comparison to a salinomycin containing diet. Nevertheless, a previous study from the same research group also failed in showing improved growth response of broilers fed 0.05 to 0.2% of several insect meals, including TM (Józefiak et al., 2018).

In experiment 1, FI of birds on TM2.0 was significantly higher than the NC on wk 2, wk 4, and wk 5 ($P < 0.05$). During the starter period, FI was 76 g greater for TM2.0 than NC ($P < 0.05$). In the overall period, FI was 160 g higher for TM2.0 (3,958 g vs. 3,798 g, $P < 0.05$), a difference of 4.2%. Feeding TM2.0 resulted in significantly ($P < 0.05$) improved FCR on wk 2 compared to NC, but it did not result in significant difference in the total period. Different results were observed in experiment 2. The difference in FI between TM2.0 and NC was not significant ($P > 0.05$), but FCR was improved for broilers fed TM2.0 (1.108 vs. 1.147, $P < 0.05$).

FI was similar for PC and TM2.0; but at the end of the trial the broilers fed the antimicrobial additives had a better FCR than TM2.0 (1.374 vs. 1.410, $P < 0.05$). In experiment 2, the use of antimicrobial additives in the broilers diets improved BWG, FI and FCR relative to NC at the end of the feeding trial ($P < 0.01$).

Table 4. Bacteria counts in ceca contents of broiler chickens fed 0.5 or 2% of tenebrio full-fat meal or antimicrobial additives (experiment 1, 1−35 d).

	Treatments[1]					
Items[2]	NC	PC	TM0.5	TM2.0	SEM[3]	*P* value
E. coli	8.31	8.44	8.54	8.80	0.185	0.315
Enterococcus spp.	4.15	4.07	4.48	4.62	0.213	0.222

[1]NC = negative control; PC = NC + 10 mg/kg enramycin + 66 mg/kg salinomycin; TM0.5 = NC + 0.5% *Tenebrio molitor* full-fat meal and TM2.0 = NC + 2.0% *Tenebrio molitor* full-fat meal.

[2]$\log_{10}$ count/g cecal contents.

[3]SEM: standard error of the mean.

However, the results for TM2.0 did not differ of PC for growth variables ($P > 0.05$). The mortality percentage was low in experiment 1 (between 0.5 and 2.1%, $P > 0.05$, data not shown), and there was no mortality in experiment 2.

In experiments 1 and 2 the same feed formulation and the same batch of insect meal were used. It was found an increased FI for the diet TM2.0 in experiment 1, but not in experiment 2 reported here. The greater FI in the first trial (3,958 vs. 3,798 g, $P < 0.05$) at 35 d was one of the reasons for the increase in BWG, because FCR was not affected by the TM2.0 diet compared to NC. Even considering the low inclusion of TM in the diet, the demonstrated preference for the insect meal (Nascimento Filho et al., 2020) may explain the higher FI. This effect on FI has been observed in other reports (Biasato et al., 2017; Islam and Yang, 2017; Benzertiha et al., 2020). When the efficiency of feed utilization is improved by feeding insect meal, as indicated by a better FCR, the FI may not be affected, as observed in experiment 2; this finding corroborates the reports by Ballitoc and Sun (2013), Bovera et al. (2016), and Islam and Yang (2017). In experiment 2, TM2.0 resulted in a significantly better FCR (−0.039, or 3.4%, $P < 0.05$).

Bacterial Counts

Nutraceutical properties of insect meal may thrive a variety of effects, including the reduction of intense inflammatory processes. This effect may minimize reduction in FI and lead to economy of nutrients. In both experiments, the PC diet resulted in the best performance among all treatments, an indication that the experimental conditions allowed for the expression of the effects of the AGP + ionophore utilized. The microbicidal activity exerted by the AMP synthetized by the insects and/or chitin and chitosan content of larvae may be directly related to the improvement in the performance of the broilers fed tenebrio meal.

Despite the possible antimicrobial effect of insect meal, in this study there was no reduction in the counts of *E. coli* and *Enterococcus* spp. in the cecal contents of the chickens ($P > 0.05$, Table 5). Different results were found by Islam and Yang (2017) feeding diet containing 0.4% TM; after challenging birds with *E. coli* and *Salmonella* Enteritidis, they observed lower cecal counts of the bacteria, and attributed this effect to the chitin and chitosan present in the exoskeleton of the larvae of tenebrio. More recently, Sedgh-Gooya et al. (2021) also observed a reduction in the counting of *E. coli* in the cecum of broilers, but only with 5.0% of TM inclusion, and not with 2.5%. They also attributed this result to chitin/chitosan.

In addition to the possible effect of TM in reducing pathogenic bacteria in the intestinal tract due to its nutraceutical properties, it may also modulate the intestinal microbiota. Biasato et al. (2019) evaluated the microbiota of broilers fed diets containing 0, 5, 10, or 15% of TM and observed an increase in bacteria of the genera *Clostridium, Alistipes*, and *Sutterella*, indicating a positive influence on the cecal microbiota; however, they observed a reduction of *Ruminococcus*, which is directly related to the production of butyrate. In another study, Józefiak et al. (2020) added 0.3% of TM in the feed and observed an increase in the abundance of the family Ruminococcacea in the ceca. They concluded that the insect meals may

Table 5. Growth performance of broiler chickens fed diets containing antimicrobial additives or 0.5 or 2% of full-fat tenebrio larvae meal (experiment 2, 1−21 d).

	Treatments[1]					
Item[2]	NC	PC	TM.05	TM2.0	SEM[3]	*P* value
1−7 d						
BWG, g	166	175	171	166	4.99	0.286
FI, g	158	168	164	158	4.41	0.106
FCR, g:g	0.949	0.961	0.959	0.954	0.011	0.892
7−14 d						
BWG, g	356[b]	385[a]	361[ab]	363[ab]	8.46	0.020
FI, g	397	418	404	408	7.21	0.061
FCR, g:g	1.110[ab]	1.087[a]	1.136[b]	1.125[ab]	0.013	0.045
14−21 d						
BWG, g	531[c]	605[a]	545[bc]	584[ab]	12.65	0.002
FI, g	656	701	662	667	12.02	0.062
FCR, g:g	1.233[c]	1.159[ab]	1.215[bc]	1.142[a]	0.016	0.002
1−21 d						
BW, g	1082[c]	1207[a]	1109[bc]	1155[ab]	19.02	<0.001
BWG, g	1053[b]	1165[a]	1067[b]	1113[ab]	18.66	<0.001
FI, g	1208[b]	1287[a]	1230[ab]	1233[ab]	17.72	0.030
FCR, g:g	1.147[b]	1.105[a]	1.153[b]	1.108[a]	0.0097	0.003

Chicks BW in g: 42.3; 42.2; 42.3; 42.3, respectively; $P = 0.710$.

[1]NC = negative control; PC = NC + 10 mg/kg enramycin + 66 mg/kg salinomycin; TM0.5 = NC + 0.5% *Tenebrio molitor* full-fat meal and TM2.0 = NC + 2.0% *Tenebrio molitor* full-fat meal.

[2]BWG = body weight gain; FI = feed intake; FCR = feed conversion ratio (g of FI/g of BWG, g/g).

[3]SEM: standard error of the mean.

[a,b,c]Means values within a row having different superscripts are statistically different by the Tukey test ($P \leq 0.05$).

stimulate the colonization with probiotic and commensal bacteria and develop barriers against infection with pathogenic bacteria.

Innate Immune Response

The innate immune system is the first defense barrier. It is responsible for triggering responses by cellular and chemical mechanisms (Tizard, 2009), with the purpose of returning to homeostasis. In situations of intense microbial challenge, this response mechanism can divert the nutrients used for maintenance and growth, impairing the animals' zootechnical performance (Roura et al., 1992). To date, there are few studies on the innate immune system and insect feeding, thus many of the comparisons about immune response presented below are related to the use of probiotics and prebiotics in broilers feed.

The results of the immune response parameters for the birds receiving the dietary treatments and challenged or nonchallenged with LPS are presented graphically in Figure 1. Within each challenge condition, dietary treatments were compared using Tukey-Kramer test.

Lysozyme is an important bactericidal agent secreted by macrophages and polymorphonuclear leukocytes (Melnick et al., 1985). It exhibits bactericidal activity by hydrolyzing the b-1,4-glycosidic linkage between N-acetylmuraminic acid and N-acetylglucosamine of bacterial cell wall peptidoglycan and is most effective against many gram-positive bacteria (Phillips, 1996).

In this experiment, no statistically significant difference among treatments was observed for LYZ ($P > 0.05$), before and after the challenge with LPS, but after the challenge with LPS it was observed a higher enzyme activity, showing that the innate immune system of birds was active due to the challenge after 6 h. Gao et al. (2009) concluded that feed with *S. cerevisiae* increased the innate immune system of chickens after verifying higher lysozyme activity before and after *Eimeria tenella* challenge. The lack of response in LYZ in the present study may be due to sampling after 6 h and not 12 h

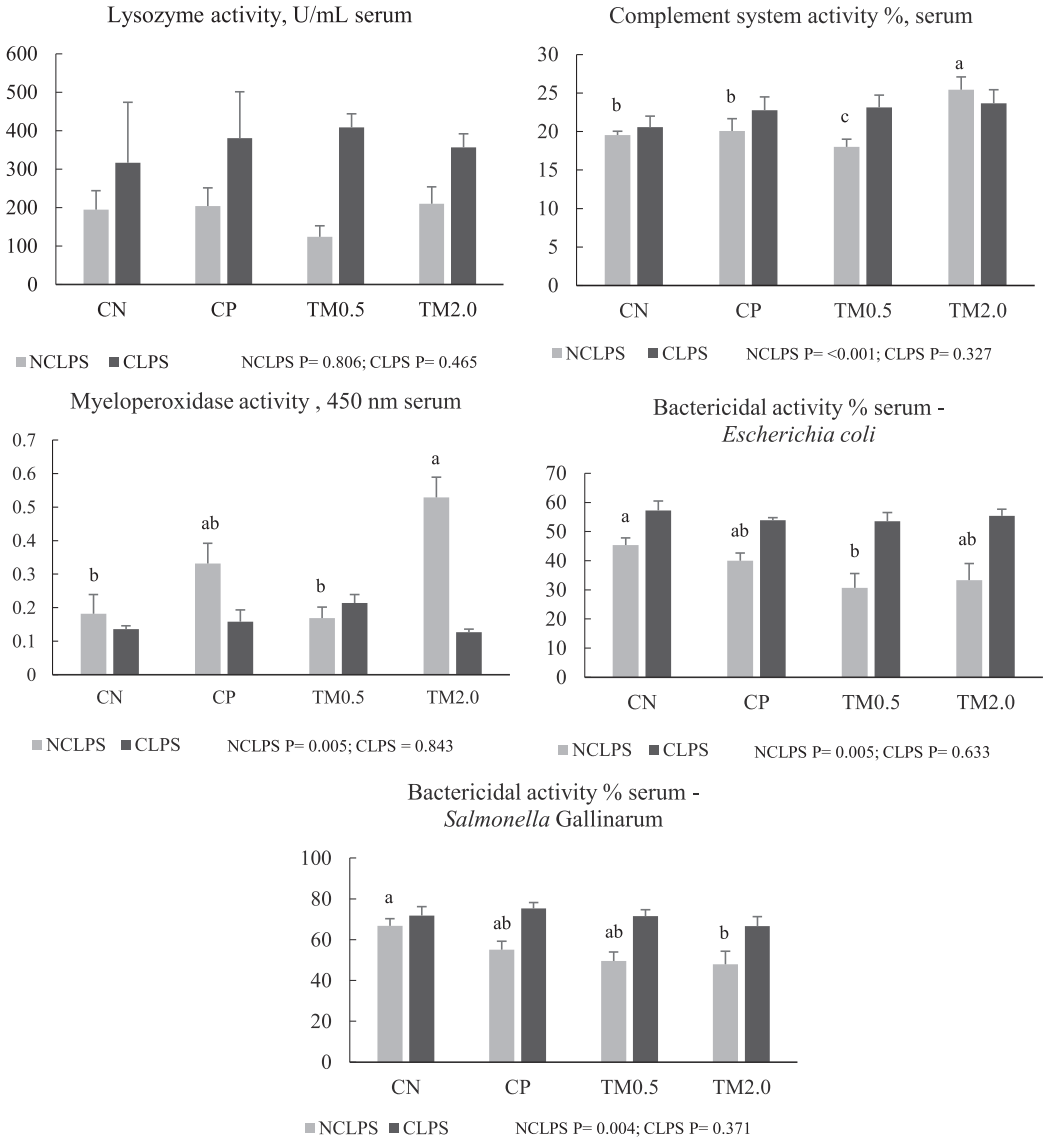

Figure 1. Serum immune response of broilers chickens fed diets containing antimicrobial additives or 0.5 or 2% of full-fat tenebrio larvae meal under LPS-induced inflammation (experiment 2, 1–21 d). NC = negative control; PC = NC + 10 mg/kg enramycin + 66 mg/kg salinomycin; TM0.5 = NC + 0.5% *Tenebrio molitor* full-fat meal and TM2.0 = NC + 2.0% *Tenebrio molitor* full-fat meal. CLPS: chickens were injected with LPS (1.43 mg/kg BW); NCLPS: chickens were injected with sterile saline solution 0.9%. Samples were collected 6 h after injection. Results from 2-way ANOVA and Tukey-Kramer test among treatments into NCLPS and CLPS groups separately, $P < 0.05$. a–c: means among NCLPS chickens' group with different letters are statistically different.

(Tizard, 2009). Thus, it is still possible that chitin present in the exoskeleton of insects may act as a prebiotic. Increased LYZ is associated with increased destructive activity of phagocytic cells (Kreukniet et al., 1995).

After LPS challenge, no treatment differences were detected ($P > 0.05$) for the immune parameters studied. However, in NCLPS broilers, TM2.0 resulted in greater HACS ($P < 0.05$) and MPO activity ($P < 0.05$) than the control (NC). Compared to the PC, the TM2.0 birds had higher HACS.

One of the most important responses of the innate immune system is the complement

system, formed by about 35 proteins that are activated in a cascade (Dunkelberger and Song, 2010) and are responsible for opsonization, attracting phagocytic cells and activating cells to release cytokines and chemokines, and inducing an inflammatory response, and act as an antimicrobial agent (Hawlisch and Kohl, 2006). In this experiment, it was determined an increase in the HACS in birds NCLPS and fed with 2.0% TM; this result provides evidence that TM can help modulate the innate immune response of broilers. However, after the challenge with LPS, no statistically significant difference of treatments was found, possibly due to the collection time, which was only 6 h.

Benzertiha et al. (2020) reported that feeding broilers with 0.2 or 0.3% of TM resulted in improved performance and also a reduction in the level of IgM and a significant increase in IL-2 and TNF-α; they attributed this effect to a possible relationship with AMP produced by insects. The activation potential attributed to insect meals may be related to the exoskeleton formed by chitin, which can trigger an immune response after recognition by the *toll-like receptor* (Komi et al., 2018).

Myeloperoxidase also plays an important role in the immune system; it is a bactericidal enzyme that produces reactive oxygen species, destroying intracellular pathogens (Khan et al., 2018). Myeloperoxidase is produced by cells responsible for the individual's first line of defense after an injury suffered, innate immunity, such as monocytes, macrophages (Nicholls and Hazen, 2005) and neutrophils (Khan et al., 2018). In this experiment, the MPO was increased in NCLPS birds fed 2.0% TM, but after the challenge with LPS, the MPO was reduced in all treatments, with no statistically significant difference ($P > 0.05$).

The chickens in PC had MPO similar to TM2.0 ($P > 0.05$). BA E. coli of TM2.0 did not differ from NC, but BA Salmonella Gallinarum of TM2.0 was inferior to NC ($P < 0.05$). BA of the serum is an important indicator of the immunity of chickens. However, although broilers fed 2.0% TM in this study showed higher activity of HACS and MPO in NCLPS ($P < 0.05$), BA of E. coli and Salmonella Gallinarum was lower than for the birds from NC and PC ($P < 0.05$). After the challenge, all treatments increased their potential for BA, but there were no statistically significant differences between treatments.

CONCLUSIONS AND APPLICATIONS

1. The benefits of using TM as a functional ingredient compared to antimicrobial additives (enramycin and salinomycin) in diets for broiler chickens were highlighted in this study. The indication of TM as a functional ingredient is ascertained by the growth improvement and innate immune response modulation in chickens fed the diet containing 2.0% TM.
2. Broiler chickens fed the diet containing 2.0% of tenebrio meal had a significantly higher FI and WG from 1 to 35 d.
3. Innate immune response was modulated in chickens fed the diet containing 2.0% of tenebrio meal at 21 d.
4. TM0.5 did not replicate the results obtained with TM2.0.

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DISCLOSURES

The authors declare no conflicts of interest.

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