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Research Article

Effect of turmeric powder supplementation on production performance and expression level of Toll Like Receptors (TLRs) in laying hens

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Abstract

The present study was aimed to assess the effect of supplementing turmeric powder on the production performance and expression level of toll- Like receptors (TLRs) in laying hens. One hundred and forty-four, 22-weeks old White Leghorn hens were divided into 6 treatments with 4 replications and each replicate had 6 birds. Control group (T1) was fed maize-soybean meal-based diet without antibiotic while birds in T2 group were fed basal diet with antibiotic. In treatment groups T3, T4, T5 and T6 basal diet was supplemented with turmeric powder @ 0.25%, 0.50%, 0.75%, and 1% respectively. After 16 weeks of the experimental period (end of the feeding trial), blood samples were collected from one bird per replicate, making four samples per treatment and thus a total of 24 samples were analyzed. Total RNA was isolated from blood samples by using TRIZOL method; cDNA was prepared, and the analysis of temporal differential gene expression profile of the genes was carried out using Step I plus real-time PCR system. The differential expression level of TLRs that includes TLR 2, TLR 4 and TLR 7 gene transcripts in birds was studied by using relative quantification method. The nutrigenomic expression analysis revealed that relative mRNA expression of TLR 2 of layer birds was found to be ($p<0.05$) enhanced in the treatment groups fed turmeric powder at all the four different levels (0.25, 0.50, 0.75 and 1%). The present study indicates that inclusion of turmeric powder in the diet of laying hen promoted an increase in fee intake, hen day egg production; egg weight; egg mass production and feed conversion ratio. In nutshell, experimental treatments containing turmeric powder in the layers' diet have potent immune modulating activity by showing significantly ($P<0.05$) up regulatory effect on relative mRNA expression of TLR 2 in the laying hens.

Keywords: Completely Randomized Design; Laying Hens; Turmeric; Gene Expression; Toll Like Receptors

Introduction

Poultry farming is highly susceptible to various infectious diseases and antibiotics are used to treat them. These antibiotics have been used as Antimicrobial Growth Promoters (AGP) in poultry feed worldwide for many years to improve food safety by improving an-

imal health and reducing or removing exogenous pathogens. Plants of the family Zingiberaceae have been widely used in dietary cuisines and in traditional oriental medications without any adverse effects. Turmeric, a member of this family has been extensively used for the treatment of a variety of inflammatory conditions and other diseases. The safety of turmeric and its yellow coloring agent, curcumin, are approved by many organizations and researchers [1-3]. Turmeric rhizome as such contains 6.3% proteins, 5.1% fat, 3.5% minerals and 69.4% Carbohydrates [4]. It has both oil parts as well as coloring pigment part. The coloring material is a rich source of the curcumin, bis demethoxy curcumin and demethoxy curcumin, collectively referred to as curcuminoids which act as powerful antioxidants [5]. Many scientists have reported that natural and organic supplements in the animal diets tend to have some effects on the expression levels of Toll-like Receptors [6]. reported that the relative mRNA expression levels of TLR 2, TLR 4 and TLR 7 in the peripheral blood of the broilers were found to be increased in the birds supplemented with graded levels of the garlic powder and holy basil leaf powder[7]. reported that Curcumin attenuates Concanavalin A-induced liver injury in mice by inhibition of Toll-like receptor (TLR) 2, TLR4 and TLR9 expression [8]. reported that dietary supplementation of turmeric powder to birds significantly decreased the expression level of toll like receptors as compared to the birds fed with control diets [9]. found that supplementation of curcumin (a component of turmeric) in the diets of humans affect the expression of toll like receptors. Since curcumin has been shown to affect the expression of many genes, he first screened several groups of TLRs that may be involved in the recognition of invading pathogens in monocytes and neutrophils. The mRNA expression levels of TLRs in curcumin treated human monocytic THP-1cells and neutrophilic-differentiated HL-60 cells were analyzed using semi-quantitative RT-PCR in this screening. Significant changes were seen only in mRNA level of TLR2 [10]. concluded that the supplementation of broiler chicken diet with yeast derived macromolecules has shown the possible role of yeast extract as a nutritional supplement to enhance gut health in chickens' possible modulation in epithelial cell turnover as well as immunomodulation. Supplementation of broiler diets with Yeast derived macromolecules resulted in both local and systemic immune responses where mainly TLR2 was involved locally, whereas only TLR4 was involved systemically with the production of both pro-inflammatory and anti-inflammatory cytokines. With all this prevailing information, the present study is aimed to assess the effect of supplementing turmeric powder on the expression level of toll- Like receptors (TLRs) in laying hens.

Materials and Methods

Birds, Diets and Experimental Design

Ethical Approval

The experimentation with the birds was conducted in accordance with guidelines approved by the Institutional Animal Ethics Committee, 12/CPCSEA Dated 6.2.2017 in the Department of Animal Nutrition, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar.

Location

The study was carried out at poultry farm, Department of Animal Genetics and Breeding, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar.

Housing

The laying hens were reared in deep litter system at poultry farm, Department of Animal Genetics and Breeding, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. The cages, feeders and waterers were regularly cleaned to maintain hygienic conditions. Proper ventilation was provided and a photoperiod of sixteen hours per day was provided.

Feeding and Watering

The hens were offered feed and water *ad libitum* through linear feeder and waterer. Waterers were located above to the feeders and they were regularly cleaned to prevent the chance of any contamination. To achieve the envisaged objectives, the comprehensive statements of the experimental methods and materials followed are given below.

Experimental Design

The experiment was planned to follow Completely Randomized Design (CRD) at uniform and standard management practices.

Birds

A total of one hundred and forty-four single comb White Leghorn hens of commercial strain, 22 weeks of age, in the first phase of their production cycle with an average weight of 1764 g were randomly divided in to six treatment groups, having four replications with six birds in each replication. Hens were fed the experimental diet for sixteen weeks of experimental period beginning at 22 weeks of age and continuing up to 38 weeks of age.

Procurement of Feed Ingredients

All the feed ingredients were procured in one lot before the start of the experiment. The feed ingredients, feed additives and supplements used were maize, groundnut cake, soybean meal, rice polish, fish meal, mineral mixture, common salt, shell grit and vitamins. The sources, composition and mixing rate of feed additives/ supplements used in ration formulations are presented in Table 2 .

Analysis of Feed Ingredients

Feed ingredients used in the diet formulations were analyzed for the proximate nutrients [11]. The chemical composition of different feed ingredients is presented in Table 1.

Experimental diets

The basal diet of laying hens was formulated as per [12]. The ingredient composition and chemical composition of the control ration (T_1), has been given in Table 2.

Table 1: Chemical composition (%DM basis) and metabolizable energy (ME, Kcal/Kg) of feed ingredients used formulating the experimental diets.

Ingredients	CP	CF	EE	Ash	OM	NFE	ME*
Maize	9.1	2.65	3.39	2.5	97.5	82.36	3309
Groundnut cake	40.9	8.9	7.94	4.52	95.48	37.74	2596
Soybean Meal	45.15	3.78	3.43	6.93	93.07	42.71	2230
Rice Polish	12.2	4.69	14.78	12.83	87.17	57.5	2737
Fish Meal	48.15	2.05	5.3	22.43	77.57	22.07	2240

*Calculated value (Singh and Panda, 2002)

Table 2: Ingredient and chemical composition of ration for layers of control group.

Feed ingredients	Percentage (%)
Maize	58
Groundnut cake	10
Soybean Meal	12
Rice Polish	9
Fish Meal	6
Mineral Mixture	1.5
Salt	0.5
Shell Grit	3
Chemical composition	**% DM basis**
CP	18.04
CF	4.34
EE	3.61
NFE	66.21
Ash	7.8
Metabolizable energy (Kcal/Kg)	2697.17

*Feed additive included Intermix Regular 10 g, Meriplex d s 10 g, Toxinil 100g per 100 Kg of ration.

Treatments

T_1:- Basal diet without antibiotics.

T_2:- Basal diet with antibiotics

T_3:- Basal diet + Turmeric Powder @ 0.25%

T_4:- Basal diet + Turmeric Powder @ 0.50%

T_5:- Basal diet + Turmeric Powder @ 0.75%

T_6:- Basal diet + Turmeric Powder @ 1.00%

Feed additives and supplements were premixed and then mixed with weighed quantity of feed ingredients to make a homogenous mixture of rations.

Production Parameters

Body Weights

Body weights of individual birds were taken at the start of the experiment and end of the experiment.

Feed Intake

Group wise feed consumption per bird was taken at interval of each 2 weeks for 8 experimental periods and for cumulative 1 - 8 periods. The feed consumption per bird for each period from 22 - 38 weeks was calculated as below.

Total amount of feed consumed during the period

$$\text{Feed intake / hen / day} = \frac{\text{Total amount of feed consumed during the period}}{\text{Total no. of hen days over the period}}$$

Per Cent Hen Day Egg Production

Egg production were recorded daily, separate record for individual bird were maintained for entire experimental period i.e. 22-38 weeks of age of laying hens. Per cent hen day egg production was calculated by using following formula:

Total no. of eggs produced during the period

$$\text{Per cent hen day egg production} = \frac{\text{Total no. of eggs produced during the period}}{\text{Total no. of hen days during the period}} \times 100$$

Egg Weight

At the end of each week the egg weights were recorded. Egg weights were measured by using electronic weighing balance. Average egg weight of each treatment was calculated as under:

$$\text{Average egg weight (g)} = \frac{\text{Weights of all eggs of the treatment}}{\text{Total no. of eggs taken for weighing}}$$

Egg Mass Production

Egg mass production was calculated using following formula:

$$\text{Egg mass production (g/day)} = \frac{\text{Per cent hen day egg production}}{100} \times \text{weight of egg}$$

Feed Conversion Ratio

Feed conversion ratio as a measure of feed efficiency was calculated in terms of feed required to produce a dozen of eggs and one kg egg mass by each group during the different experimental periods and cumulative from 22 - 38 weeks of age of laying hens.

$$\text{Feed intake per dozen eggs} = \frac{\text{Feed consumed (kg) by the group during the period}}{\text{Feed consumed (kg) by the group during the period}}$$

$$\text{Feed intake per kg of egg mass} = \frac{\text{Feed consumed (kg) by the group during the period}}{\text{Egg mass (kg) produced during that period}}$$

Expression Level of Toll Like Receptors (Tlrs)

Blood collection and analysis

At the end of the feeding trial, blood samples were collected from one bird per replicate, making four samples per treatment and thus a total of 24 samples were analyzed. About 2 ml of blood was collected from each bird via brachial wing vein puncture using sterilized syringes and 5 ml scalp vein needle set into vacutainer containing Ethylene Diamine Tetraacetic Acid (EDTA) for TLR mRNA expression. Plasma was prepared by centrifuging the blood at 3000 rpm for 10 min. The plasma was then transferred into a micro centrifuge tube using a Pasteur pipette and stored at -20°C until further analysis.

Reverse transcription (cDNA synthesis); RNA extraction and preparation of cDNA

Total RNA was isolated from blood samples by using TRIZOL® as per the manufacturer's instruction. In brief, 1 ml of TRIZOL® reagent, 200 µl of chloroform was added to 600 µl of blood followed by centrifugation for phase separation and precipitation with isopropanol. Total RNA extracted was dissolved in 20 µL NFW and quantified using Qubit® 2.0 fluorometer (Invitrogen). Reverse transcription was carried out with total reaction volume of 20 µL using cDNA synthesis kit (Promega A5000). Briefly, NFW (7.3 µL), 5X RT buffer (4 µL), $MgCl_2$ (1.2 µL), 10 mM dNTPs (Promega A5000) (1 µL), total RNA (5 µL), Random hexamer (1 µL), RNAase in (0.50 µL). The polymerase chain reaction (RT-PCR) cyclic conditions were as initial incubation at 25°C for 5 min, reverse transcription at 42°C for 1 h, extension temperature is optimized between 37 - 55ºC and deactivation at 70°C for 15 min in thermal cycler (Applied Biosystem). The cDNA was stored at −20°C till further use (Tables 3-7).

Table 3: Mean values of feed consumption (g/hen/day) during progressive age (weeks) under different dietary treatments.

Weeks/ Treatment	T_1	T_2	T_3	T_4	T_5	T_6
22-24	110.30 ^{a}±0.49	110.16 ^{a}±1.21	110.23 ^{a}±1.46	111.56 ^{a}±1.26	106.63^{b}±0.50	102.85^{c}±1.31
24-26	128.63 ^{a}±2.16	125.95 ^{a}±2.02	126.60 ^{a}±1.14	125.24 ^{a}±1.26	116.57^{b}±1.55	115.76^{b}±1.48
26-28	116.21^{ab}±1.73	117.70^{ab}±1.15	118.48^{ab}±1.75	120.48 ^{a}±1.74	114.69^{b}±0.38	114.83^{b}±0.77
28-30	118.02 ± 4.10	119.02 ± 3.88	120.37 ± 2.57	119.35 ± 1.84	117.10 ±1.58	116.21 ±0.98
30-32	118.79 ± 3.25	116.12 ± 1.49	119.45 ± 1.57	117.38 ± 0.97	121.75 ±1.55	117.80 ±1.32
32-34	120.37 ± 1.62	118.07 ± 1.35	121.37 ± 1.65	122.26 ± 1.27	115.33 ±2.66	118.67 ±1.73
34-36	121.57 ± 1.57	119.46 ± 1.14	122.65 ± 0.33	124.57 ± 2.16	123.67 ±1.00	126.71 ±4.00
36-38	125.9 ± 1.93	124.04 ± 1.79	126.19 ± 1.59	127.13 ± 1.27	125.56 ±1.47	129.05 ±1.77
Mean	119.98 ± 2.11	118.81 ± 1.70	120.67 ± 1.82	121.00 ± 1.77	117.66 ±2.12	117.74 ±2.90

The mean values in same row with different superscripts differ significantly (P< 0.05)

Table 4: Mean values of per cent hen day egg production during progressive age (weeks) under different dietary treatments.

Weeks/ Treatment	T_1	T_2	T_3	T_4	T_5	T_6
22-24	63.57 ^{c}±1.22	62.06^{bc}±2.18	56.24^{ab}±4.22	63.68^{c}±0.34	56.74^{ab}±0.99	54.63 ^{a}±1.66
24-26	71.61^{ab}±1.70	67.16 ^{a}±1.38	69.53^{ab}±2.74	73.14^{b}±0.37	68.84^{ab}±1.37	69.53^{ab}±1.80
26-28	66.41 ^{a}±0.97	72.81^{ab}±2.53	72.31^{ab}±2.93	74.53^{b}±1.61	71.12^{ab}±1.02	67.25 ^{a}±2.76
28-30	58.65 ^{a}±0.43	63.90 ^{b}±1.11	63.27 ^{b}±2.53	67.01^{b}±2.03	67.97 ^{b}±0.50	67.78 ^{b}±1.45
30-32	62.03 ^{a}±1.17	66.37^{ab}±2.42	71.42 ^{b}±1.37	68.74^{b}±2.97	67.55 ^{b}±1.63	68.29 ^{b}±1.61
32-34	61.03 ^{a}±1.90	70.13 ^{b}±2.46	71.72 ^{b}±2.85	73.98^{b}±1.93	72.92 ^{b}±2.68	69.82 ^{b}±2.43
34-36	58.14 ^{a}±0.75	67.43 ^{b}±2.70	69.24 ^{b}±2.62	71.12^{b}±2.14	70.50 ^{b}±1.40	73.82 ^{b}±2.18
36-38	58.31 ^{a}±1.22	65.07 ^{b}±1.98	68.36 ^{b}±1.74	68.15^{b}±1.02	68.70 ^{b}±1.44	69.06 ^{b}±1.64
Mean	62.75 ^{a}±1.58	66.87^{ab}±1.21	67.76^{ab}±1.93	69.86^{b}±1.75	68.05 ^{b}±1.74	67.52^{ab}±1.97

The mean values in same row with different superscripts differ significantly (P< 0.05)

Table 5: Mean values of egg mass production (g/day/hen) during progressive age (weeks) under different dietary treatments.

Weeks/ Treatment	T_1	T_2	T_3	T_4	T_5	T_6
22-24	33.99^{bc}±0.50	33.05^{abc}±1.18	30.65^{ab}±1.89	34.85^{c}±0.64	30.92^{ab}±1.16	29.85 ^{a}±0.91
24-26	38.92^{ab}±0.85	36.46 ^{a} ±1.49	37.73^{ab}±0.87	40.06^{b}±1.04	37.49^{ab}±1.32	38.18^{ab}±0.76
26-28	36.35 ^{a}±0.50	40.12 ^{ab}±1.44	39.74^{ab}±1.53	40.95^{b}±0.93	39.35^{ab}±0.93	37.27^{ab}±1.60
28-30	32.19 ^{a}±0.23	35.06 ^{ab}±0.30	34.92^{ab}±1.62	37.47^{b}±1.18	37.53 ^{b}±0.60	37.48 ^{b}±0.92
30-32	34.33 ^{a}±0.67	37.09 ^{ab}±1.11	40.02 ^{b}±0.80	39.31^{b}±1.11	37.75 ^{b}±1.12	38.17 ^{b}±0.85
32-34	34.76 ^{a}±1.07	39.91 ^{b} ±1.36	41.15 ^{b}±1.62	42.32^{b}±0.76	41.37 ^{b}±1.39	39.65 ^{b}±1.33
34-36	33.41 ^{a}±0.32	39.07 ^{b} ±1.70	40.75 ^{b}±1.72	41.86^{b}±1.18	40.68 ^{b}±0.80	42.36 ^{b}±1.32
36-38	34.02 ^{a}±0.50	38.32 ^{b} ±1.25	40.68 ^{b}±1.01	40.50^{b}±0.50	40.49 ^{b}±1.08	40.76 ^{b}±0.93
Mean	34.77 ^{a}±0.72	37.38 ^{ab}±0.87	38.20 ^{b}±1.30	39.67^{b}±0.88	38.20 ^{b}±1.17	37.96^{ab}±1.31

The mean values in same row with different superscripts differ significantly (P< 0.05)

Table 6: Mean values of feed intake (kg) per dozen egg production during progressive age (weeks) under different dietary treatments.

Weeks/ Treatment	T_1	T_2	T_3	T_4	T_5	T_6
22-24	2.08^{a} ±0.04	2.14^{ab}±0.09	2.39^{b} ±0.17	2.10^{ab}±0.01	2.25^{ab}±0.05	2.24^{ab}±0.09
24-26	2.16^{ab}±0.06	2.25^{b} ±0.07	2.18^{ab}±0.09	2.05^{ab}±0.02	2.03^{a} ±0.04	2.22^{a} ±0.06
26-28	2.09 ± 0.04	1.95 ± 0.09	1.97 ± 0.07	1.96 ± 0.04	1.94 ± 0.03	2.05 ± 0.07
28-30	2.36 ^{c}±0.05	2.29^{bc}±0.08	2.39^{c} ±0.10	2.15^{ab}±0.06	2.06^{a} ±0.03	1.98^{a} ±0.03
30-32	2.24^{c} ±0.02	2.09^{ab}±0.05	2.01^{a} ±0.02	2.05^{ab}±0.05	2.17^{bc}±0.05	2.07^{ab}±0.04
32-34	2.27^{b} ±0.08	2.03^{ab}±0.16	2.05^{ab}±0.19	2.03^{ab}±0.17	1.87^{a} ±0.10	2.02^{ab}±0.14
34-36	2.37^{b} ±0.08	2.19^{ab}±0.18	2.18^{ab}±0.16	2.12^{a} ±0.13	2.10^{a} ±0.08	2.06^{a} ±0.08
36-38	2.36 ± 0.08	2.29 ± 0.15	2.25 ± 0.18	2.24 ± 0.09	2.18 ± 0.17	2.26 ± 0.08
Mean	2.24^{b} ±0.04	2.15^{ab}±0.04	2.18^{ab}±0.06	2.08^{a} ±0.03	2.07^{a} ±0.04	2.09^{a} ±0.04

The mean values in same row with different superscripts differ significantly (P< 0.05)

Table 7: Mean values of feed intake (Kg) per kg egg mass production during progressive age (weeks) under different dietary treatments.

Weeks/ Treatment	T_1	T_2	T_3	T_4	T_5	T_6
22-24	3.25 ± 0.06	3.35 ± 0.16	3.63 ± 0.23	3.20 ± 0.03	3.46 ± 0.15	3.45 ± 0.13
24-26	3.31^{ab}±0.08	3.47^{b} ± 0.17	3.36^{ab}±0.11	3.12^{ab} ±0.04	3.12^{ab}±0.11	3.03^{a} ±0.09
26-28	3.20 ± 0.09	2.94 ± 0.13	2.99 ± 0.10	2.94 ± 0.13	2.91 ± 0.07	3.09 ± 0.12

28-30	3.67^{d} ±0.10	3.39^{bcd}±0.11	3.46^{cd}±0.12	3.19^{abc}±0.10	3.12^{ab}±0.04	3.00^{a} ±0.10
30-32	3.43^{c} ±0.06	3.14^{ab} ±0.06	2.98^{a} ±0.04	2.99^{b} ± 0.24	3.22^{bc}±0.09	3.09^{ab}±0.05
32-34	3.47^{b} ±0.10	2.97^{a} ± 0.11	2.96^{a} ±0.11	2.89^{a} ± 0.08	2.79^{a} ±0.10	3.00^{a} ±0.14
34-36	3.64^{b} ±0.06	3.07^{a} ± 0.11	3.02^{a} ±0.12	2.98^{a} ± 0.11	3.04^{a} ±0.61	2.99^{a} ±0.11
36-38	3.70^{b} ±0.10	3.24^{a} ± 0.12	3.10^{a} ±0.10	3.14^{a} ± 0.06	3.11^{a} ±0.11	3.32^{a} ±0.16
Mean	3.46^{b} ±0.07	3.19^{a} ± 0.07	3.19^{a} ±0.10	3.06^{a} ± 0.04	3.10^{a} ±0.07	3.12^{a} ±0.06

The mean values in same row with different superscripts differ significantly (P< 0.05)

Real Time PCR

For the analysis of temporal expression profile of different genes, real-time PCR was carried out using Step I plus real-time PCR system. For the real-time PCR reaction, SYBR Green dye-based PCR master mix (Affymetrix) was used, and all the instructions were followed as per the manufacturer. Table 8 represents the oligonucleotide sequences of sense and antisense primers. The reaction for the target gene, TLRs (TLR 2, TLR 4, and TLR 7), and the endogenous control, β-actin gene was carried out in triplicate along with non-template control as a negative control for each sample. The reaction mixture used to carry out the real-time PCR reaction for TLRs 2, 4 and 7; and β-actin gene contains 2X SYBR green PCR master mix (Affymetrix, 12.5 µL), primers (forward and reverse 0.50 M each), NFW (variable), and template (3 µL). The cyclic conditions used for amplification were according to the instructions of the manufacturer. Amplification was done with denaturation for 15 min at 95°C, followed by 40 cycles of denaturation for 5 s at 95°C, and annealing/elongation for 30 s at 60°C, and a final melting curve analysis.

Relative Quantitation by Comparative CT Method (ΔΔCT Method)

The average CT (Threshold cycle) value obtained for the TLRs 2, 4 and 7 (target) gene was normalized to β-actin (endogenous control). The data obtained were subjected to comparative CT method for the analysis of the expression levels of targeted TLR gene and endogenous control. The sample at 26 h of incubation was selected as calibrator.

Sequencing of Product

Amplicons were sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) on an automatic ABI 3130 xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The sequence obtained shows 100% nucleotide identity with the TLR sequence of chicken available in the global database.

Statistical Analysis

The resultant data were statistically analysed according to the procedure laid down by [13].

Results

Feed Intake

The data pertaining to average feed intake (g) per bird per day during progressive age of layers in different dietary treatments are presented in Table 3. The mean values of feed consumption of layers from 22 to 38 weeks of age were 119.98, 118.81, 120.67, 121.00, 117.66 and 117.74 g per bird per day in treatment groups T_1, T_2, T_3, T_4, T_5 and T_6, respectively. The results of the study depicted that there was no significant (P<0.05) difference among different dietary treatments, showing that feeding of different levels (0.25%, 0.50%, 0.75% and 1%) of turmeric powder did not affect feed consumption (g/hen/day). The mean value of feed consumption was minimum in T_5 andT_6 and maximum in T_4. Feed intake during week 22-24 was maximum in T_4 treatment followed by T_1, T_3, T_2, T_5, and T_6 and significant (P<0.05) difference among treatments T_5 and T_6 was observed as compared to T_1, T_2, T_3 and T_4. Study during experiment showed that during 3 periods (22-24, 24-26 and 26-28 week) feed intake decreased significantly (P<0.05) in treatment groups T_5 and T_6 as compared to T_1, T_2, T_3 and T_4. During periods (28-30, 30-32, 32-34, 34-36 and 36-38 week) there was no significant difference in feed consumption among different treatment groups. Thus, it can be concluded that dietary supplementation of turmeric powder at different levels has no effect on feed intake.

Hen Day Egg Production

The per cent hen day egg production, for the 8 periods (22-24, 24-26, 26-28, 28-30, 30-32, 32-34, 34-36 and 36-38 weeks of age) of 2 weeks each and cumulative production of 1-8 periods (22-38 weeks), are presented in Table IV. The cumulative hen day egg production values were 62.75, 66.87, 67.76, 69.86, 68.05 and 67.52 % in treatment groups T_1, T_2, T_3, T_4, T_5 and T_6, respectively. The results of the study unveiled that groups fed diets at 0.50 and 0.75% turmeric powder had significantly (P<0.05) higher hen day egg production than that of negative control. But, there was no significant difference in hen day egg production in groups fed different dietary levels of turmeric powder as compared to T_2. The results of study during 22-24, 24-26 and 26-28 weeks indicated that, when diet of layers was supplemented with 0.75 and 1.0% of the turmeric powder there was (P<0.05) negative effect on per cent hen day egg production in comparison to negative and positive control groups. After 3 periods hen day egg production was significantly (P<0.05) decreased in T_1 as compared to other treatment groups. The minimum hen day egg production (ranged from 54.63% in T_6) was found at age of 22-24 weeks of age and maximum production (74.53 % in T_4) was found at 26-28 weeks of age. In nutshell, the data of the study revealed that feeding of hens with turmeric

powder at different dietary levels had significant (P<0.05) positive effect on per-cent hen day egg production in treatment group T_4 and T_5 (0.50 and 0.75% turmeric powder) as compared to negative control group.

Egg Mass Production

The results of egg mass production (g) per bird per day under different dietary treatments during progressive weeks of age are illustrated in Table V. The results of the research have shown that egg mass production make a particular increasing trend during different periods of age as the turmeric powder level increased. The minimum egg mass production was at 22-24 weeks of age and maximum at 32-34 weeks of age in different dietary treatments. The collective egg mass production (22-38 weeks) values were 34.77, 37.38, 38.20, 39.67, 38.20, and 37.96 g per bird per day in treatment groups T_1, T_2, T_3, T_4, T_5 and T_6, respectively. The study revealed that the egg mass production (g/day/hen) during progressive age (22-28 weeks) was increased significantly (P<0.05), where highest values were observed at all the levels of turmeric powder feeding. However, in T_6 egg mass was statistically similar to T_1 and T_2 but it was increased numerically. The results of the study divulged that egg mass production was decreased in T_1 during 26-38 weeks period as compared to other treatment groups, indicating that turmeric powder supplementation has positive effect on egg mass production in laying hens same as antibiotics. With respect to the whole period of experiment, egg mass increased significantly (P<0.05) at different dietary levels of turmeric powder as compared to negative control.

Feed Conversion Ratio

Feed conversion ratio in terms of Kg feed per dozen egg production and Kg feed per Kg egg mass production were used as a measure of efficiency of utilisation of feed for egg production. The FCR values of progressive weeks of age and cumulative (22-38weeks) are presented in Table IV and V, respectively.

Feed Intake Per Dozen Egg Production

The mean values of feed consumption per dozen egg production of layers from 22 to 38 weeks of age were 2.24, 2.15, 2.18, 2.08, 2.07 and 2.09 Kg in treatment groups T_1, T_2, T_3, T_4, T_5 and T_6, respectively Table 4. The results of study revealed that the feed consumption was significantly (P<0.05) increased in the treatment T_4, T_5 and T_6 (0.50, 0.75 and 1% turmeric powder, respectively) as compared to the negative control T_1, however, it was insignificant as compared to other treatment groups during entire length of the experiment. During third and last period feed intake per dozen egg production did not differ significantly (P<0.05) among different treatment groups. Comparatively overall minimum values of feed intake per dozen egg production was observed in treatment T_4, T_5 and T_6. The mean values of feed intake per dozen egg production was lowest in T_1 during the entire length of experiment as compared to negative control. Thus, it can be concluded that turmeric powder has positive effect on feed conversion efficiency in laying hens.

Feed Intake Per Kg Egg Mass Production

The data pertaining to feed intake (kg) per kg egg mass production during progressive weeks of age and cumulative means are presented in Table 7. The cumulative mean values (22-38 weeks) of feed consumption per kg egg mass production were 3.46, 3.19, 3.19, 3.06, 3.10 and 3.12 Kg in treatment groups T_1, T_2, T_3, T_4, T_5 and T_6, respectively. The results of study revealed that the feed consumption per kg egg mass during the experiment decreased significantly (P<0.05) in different treatment groups fed with turmeric powder as compared to negative control groups. The values of feed intake per kg egg mass production differ significantly (P<0.05) among the laying hens of different treatment groups during entire experiment except for 22-24 and 26-28 weeks, during these periods feed intake per kg egg mass production did not differ significantly among the laying hens of different treatment groups. Maximum value for feed intake per kg egg mass production was observed in T_1 during last period of the experiment and minimum value was observed in T_5 during sixth period of the experiment. It showed that the efficiency of utilisation of feed for egg mass production increased significantly (P<0.05) with addition of turmeric powder in the diet of laying hens (Table 8).

Table 8: Oligonucleotide sequences of sense and antisense primers for real-time PCR product determined.

Gene[1]	Primer	Primer sequence[2]	Accession No.	Product size
β-Actin	Sense	5′-GAGAAATTGTGCGTGACATCA-3′	L08165	152
	Antisense	5′-CCTGAACCTCTCATTGCCA-3′		
TLR 2	Sense	5′-CATTCACCATGAGGCAGGGATAG-3′	AB046533	157
	Antisense	5′-GGTGCAGATCAAGGACACTAGGA-3′		
TLR 4	Sense	5′-TTCAGAACGGACTCTTGAGTGG-3′	AY064697	131
	Antisense	5′-CAACCGAATAGTGGTGACGTTG-3′		
TLR 7	Sense	5′-TTGCTGCTGTTGTCTTGAGTGAG-3′	AJ627563	182
	Antisense	5′-AACAACAGTGCATTTGACGTCCT-3′		

[1]TLR 2 = Toll-like receptor 2; TLR 4 = Toll-like receptor 4; TLR 7 = Toll-like receptor 7.

[2]Primers for Toll-like receptors and β-actin were described by Sato *et al.* (2009) and Bai *et al*. (2008), respectively.

Expression Pattern of Toll-Like Receptors (TLRs)

The differential expression level of TLRs, viz. TLR 2, TLR 4 and TLR 7 gene transcripts in the Ven Cobb commercial broiler strains was studied by relative quantification method. The level of target mRNA in different treatment groups was determined by comparative C_T method ($\Delta\Delta C_T$ method). The nutrigenomic expression analysis as presented in Table 9 revealed that relative mRNA expression of TLR 2 of layer birds was found to be ($p<0.05$) enhanced in the treatment groups fed turmeric powder at all the four different levels (0.25, 0.50, 0.75 and 1%). In a nutshell, experimental treatments containing turmeric powder at different levels in the layers' diet have potent immune modulating activity by showing significantly ($P<0.05$) up regulatory effect on relative mRNA expression of TLR 2 and non-significant down regulation pattern of TLR 4 in the laying hens. There was a significant increase in the relative mRNA expression of TLR 2 in the plasma of the birds fed diet supplemented with different levels of turmeric powder. TLR 2 recognizes a variety of microbial components.

Table 9: Relative quantitation expression analysis of the Toll like receptors (TLR 2, TLR 4 and TLR 7) with reference to the endogenous reference gene β actin.

Sample Name	Target Name	CT Mean	CT SD	ΔCT Mean	ΔCT SE	ΔΔCT	R Q
T_1	TLR 2	26.885	0.215	5.509	0.127	0	1
T_2		26.534	0.036	5.34	0.023	-0.169	1.124
T_3		25.485	0.055	3.483	0.039	-2.026	4.073
T_4		25.626	0.113	4.026	0.134	-1.483	2.795
T_5		27.447	0.054	3.092	0.037	-2.417	5.34
T_6		27.728	0.185	3.315	0.176	-2.194	4.575
T_1	TLR4	22.358	0.041	0.982	0.034	0	1
T_2		23.074	0.07	1.88	0.042	0.898	0.5037
T_3		23.123	0.147	1.121	0.088	0.139	0.908
T_4		22.532	0.088	0.931	0.128	-0.05	1.036
T_5		25.653	0.076	1.298	0.048	0.316	0.803
T_6		25.406	0.151	0.993	0.164	0.011	0.992
T_1	TLR7	20.5091	0.198	-0.786	0.117	0	1
T_2		20.808	0.06	-0.219	0.036	0.5067	0.675
T_3		21.168	0.042	-0.834	0.034	-0.048	1.034
T_4		20.733	0.082	-0.868	0.127	-0.082	1.058
T_5		23.014	0.054	-1.341	0.037	0.5055	0.68
T_6		23.717	0.065	-0.696	0.144	-0.09	1.064
T_1	β ACTIN	21.376	0.04				
T_2		21.194	0.01				
T_3		22.002	0.04				
T_4		21.6	0.2				
T_5		24.355	0.03				
T_6		24.413	0.24				

These include lipoproteins/lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from gram positive bacteria. TLR 4 is the principal receptor for lipopolysaccharide, which is a major component of outer membrane of gram-negative bacteria. Result findings related to the relative mRNA gene expression of TLR 2 and TLR 4 in the present study, it can be stated that increased regulation of TLR 2 might be due to increased population of Lactobacillus sp. caused by dietary supplementation of turmeric powder. Similarly, non-significant down regulation of TLR 4 might be due to decreased population of E. coli caused by dietary supplementation of turmeric powder. TLR 7 family is implicated in intracellular recognition of nucleic acids. The TLR 7 recognizes some antiviral compounds and single-stranded viral RNA. In this study, supplementation of diet with turmeric powder had no significant effect on the relative mRNA expression of TLR 7 in the plasma of the laying birds. The nutrigenomic expression analysis revealed that relative mRNA expression of TLR2 was found to be ($p<0.05$) increased in the treatment groups fed turmeric powder at all the four different

levels. While, there was non-significant down regulation pattern of relative mRNA expression of TLR4 in the plasma of laying hens fed diet supplemented with turmeric powder at different levels. However, the data pertaining to the relative mRNA expression levels of TLR7 in the plasma of birds was non-significant in the different experimental groups. The treatments having turmeric powder in the layers' diet recorded potent immune modulating activity by showing significantly (P<0.05) up regulatory effect on relative mRNA expression of TLR2 and non-significant down regulation pattern of TLR4 in the laying hens. However, mRNA expression of TLR7 was not affected by dietary inclusion of turmeric powder.

Discussion

Feed Intake

The results of the study depicted that the average feed intake (22-38 weeks) of layers supplemented with turmeric powder show no significant (P<0.05) difference among different dietary treatments, showing that feeding different levels (0.25, 0.50, 0.75 and 1%) of turmeric powder in the diet of laying hens had no positive effect on feed intake. Intake was significantly lower when the level of turmeric powder in the diet was increased to 0.75% and above during initial periods of the experiment. It may be due to decreased palatability of feed at higher levels of turmeric powder but, after initial periods birds got adopted to the feed and feed intake became normal gradually in comparison to control groups. These findings are in full agreement with findings of [14]. who observed that feed intake in the birds fed diets containing 0.50 or 1.0% turmeric powder did not differ significantly, however the feed intake values were increased numerically. Similarly [15]. reported that feed intake was not changed by the dietary treatments, suggesting that addition of turmeric powder did not affect palatability [16]. also reported that turmeric powder supplementation up to 2 % did not affect feed intake but increasing supplementation to 4% resulted in a significant lower feed intake [17]. found that inclusion of turmeric root in the diet did not affect (P<0.05) feed intake in the birds when compared to the basal diet. Likewise [18]. also reported that given turmeric powder showed no significant difference (P<0.05) on feed intake [19]. reported that diet containing turmeric powder at 5 or 10 g/kg showed no significant increase in the feed consumption. Similarly [20]. found that there is no significant difference in feed consumption between treatment groups. Contrary to these findings [21]. reported that hens fed 1 % turmeric powder had lower feed consumption which resulted in reduction of egg production and egg mass compared with control diet. The lower egg production and egg mass might be related to the lower feed consumed by laying hens fed 1% turmeric powder.

While [22]. reported that the inclusion of turmeric mixture at levels of 0.75 % and 1 % in the diets improved feed intake. Contrary to this [23]. found that feeding turmeric (444 ppm) in the basal diet significantly decreased feed intake in birds. [24].found that birds fed turmeric at the level of 0.50% exhibited (P<0.05) decreased feed intake as compared to control group [25]. reported that the birds fed diets containing turmeric powder at levels of 1% showed significant decrease in the feed intake. This may be due to decreased palatability of feed due to higher levels of turmeric. However, birds fed diet containing turmeric powder at level of 3% did not differ significantly [26]. found hens fed diets containing 1.5 and 2 g/kg of turmeric powder had lower feed intake than the other groups [27]. reported that birds fed diet having turmeric powder at 1% level showed significant decrease in the feed intake [28-34].

Growth Performance

Perusal of the data indicated that no significant effect on hens' body weight was reported in present study in response to dietary turmeric powder supplementation as compared to the no added control (with and without antibiotic) program throughout the 16-week feeding period. Although, there was numerical increase in the body weight gain with the increasing levels of turmeric powder use (at 0.25 and 0.50 % level) but, statistically data did not differ significantly. In agreement with the results of the present trial [33]. reported that body weight gain was not affected by 0.50% turmeric powder. Similarly, [28] also reported that dietary supplementation of turmeric powder did not affect the body weight gain in laying hens as compared to the control groups. Similarly, [29]. stated that dietary supplementation of turmeric powder at different levels in laying hens did not affect the body weight gain as compared to the control groups [14]. also reported that body weight gain was numerically increased but not significantly when compared to hens fed basal diet.

Egg Production

Hens fed with turmeric powder at different dietary levels did not affect egg production significantly when compared to the positive control, but it differ significantly (P<0.05) in T_4 and T_5 as compared to negative control group. It may be due to decreased efficiency of feed utilization of birds in the absence of any growth promoters in negative control. Although, there was numerical increase in the egg production with the levels of turmeric powder (at 0.25 and 1.0 % level) but, statistically data did not differ significantly. These finding agree with the findings of [30]. who recorded increased egg production in laying hens fed with herb-derived mineral toxin binder production containing *Curcuma longa*. Confirming these findings, most studies conducted with laying hens have pointed out the positive relationship between turmeric powder supplementation and egg production rate [14]. who stated that the addition of 0.50 or 1 % turmeric significantly increased the egg production [31]. also reported that supplementation of turmeric powder, regardless of period of administration, increased the total number of egg production until 9 months of age (P<0.05). Birds fed high carbohydrate ration and supplemented with turmeric powder for 30 days prior to sexual maturity had 20% higher egg production as compared to control [15]. reported that egg production was the highest in the layers fed diet with 0.50% turmeric powder and

the lowest in the layers fed the control diet [16]. observed that turmeric powder supplementation up to 4 % in the ration of laying hen showed a significant effect to improve egg production, the improved egg production performance was apparently maintained by turmeric supplementation along the 3 periods of experiment [19]. reported that diet containing turmeric powder at 5 g/kg showed significant increase (P<0.05) in the egg production but at 1% level the egg production was not affected significantly (P<0.05). Findings of these experiment was contrary to the [20]. who observed that dietary supplementation of turmeric at 1.0 g/kg did not influence hen house egg production as well as hen day egg production. Similarly [27]. reported that feeding of turmeric at 10.0 or 30.0 g/kg did not influence egg production of laying hens [31]. also stated that there was no significant difference in egg production when lower levels of annatto extract and turmeric were added to layer diets.

Egg Weight

The perusal of the data obtained clearly indicate that turmeric powder supplementation in treatment groups fed different levels of turmeric powder had no positive effect on egg weight as compared to the positive control and negative control groups. These results are similar to the findings of [27]. who found that supplementation of turmeric at 10.0 or 30.0 g/kg did not influence egg weight of laying hens [25]. also observed that egg weight was not affected by dietary supplementation of different levels of turmeric powder. Similarly, [16]. reported that turmeric supplementation up to 4% did not affect egg weight. While, opposite to these findings [14]. reported that egg weight increased significantly after feeding turmeric at 0.50 or 1% as compared to the control diets. Similarly [15]. found that egg weight in the groups fed diets with 0.50% turmeric powder was higher than that in the other groups.

Egg Mass Production

The analysis of the data obtained clearly indicate that birds fed turmeric powder at different dietary levels did not affect egg production significantly when compared to the positive control but, it differ significantly (P<0.05) in T_3, T_4 and T_5 as compared to negative control group. Reduced per cent hen day egg production may be the reason of decreased egg mass production in negative control group. Although, there was numerical increase in the egg production in T_6 but, values were increased numerically not significantly. These results are similar to the findings of [14]. who reported that addition of turmeric at 0.50 or 1% significantly increased the egg mass when compared with the control diets [15]. reported that egg mass in the groups fed diet with turmeric powder were significantly higher than that of the control (P<0.05). [19]. reported that turmeric powder at 5 or 10 g/kg feed showed significant increase (P<0.05) in the egg mass. Contrary to the findings of experiment [27]. reported that supplementation of turmeric powder at 10.0 or 30.0 g/kg did not influence egg mass in laying hens. Similarly [26]. stated that different levels of turmeric powder had no effect on egg mass production in separate weeks, however 2 g/kg turmeric powder significantly (P<0.05) increased egg mass production over the 4 weeks assay. But the overall mean values were non-significant.

Feed Conversion Ratio

The results of present study revealed that feed consumption per dozen egg production and per kg egg mass production was improved (P<0.05) by dietary supplementation of turmeric powder at different levels as compared to negative control but results were non-significant with positive control. These findings indicate that the turmeric powder used in the present study had positive effect on efficiency of conversion of feed to egg mass same as positive control. Present findings agree with the findings of [21]. who reported that hens fed 1 % turmeric powder had lower feed conversion ratio when compared with control diets [14]. stated that feed conversion ratio in the layers fed turmeric at 0.50 or 1% was improved when compared to hens fed basal diet [27]. also reported that hens fed 1% turmeric powder had lower feed conversion ratio as compared with the control diet. Similarly [26]. observed that feed conversion ratio was affected by turmeric powder supplementation after just 2 weeks post starting the experiment. Diet containing 2 g/kg turmeric powder showed the lowest FCR in weeks 2, 3, 4. This effect was mainly due to decreased feed intake and increased egg mass production. While contrary to findings of the experiment [25]. stated that feed efficiency was not affected by dietary supplementation of different levels of turmeric powder [33] also reported that feed conversion ratio was not affected by 0.50% turmeric powder. Likewise, [20] reported that feed conversion ratio per dozen eggs differed significantly after 45 weeks of age in all treatment groups, but there was no significant difference in overall mean FCR during the experimental period.

Differential Expression of Toll-Like Receptors

The findings of our study are in consistent agreement with [6] who stated that the relative mRNA expression levels of TLR 2 in the peripheral blood of the broilers were found to be increased (P<0.05) in the birds supplemented with graded levels of the garlic powder and holy basil leaf powder as compared to the untreated group [8] reported that dietary supplementation of turmeric powder to birds significantly decreased the expression level of TLR 4 as compared to the birds fed with control diets [34]. also stated that curcumin attenuates inflammation through inhibition of TLR-4 receptor in experimental colitis in rats. TLR 4 is activated by bacterial cell wall components and provides immune protection by virtue of its ability to discriminate between self- and non-self-pathogens. This activation finally results in the activation of transcription factor NFkB which induces inflammatory cytokines, growth factors and has been implicated in the pathogenesis of IBD. Thus, inhibition of NFkB has been considered as a putative target for intervention in IBDs. However, because NFkB is induced through various pathways, identifying upstream signaling molecules shall prove a better target for managing IBD. Curcumin has been shown to ameliorate IBD conditions both in humans and experimental animals through

inhibition of NFkB. Although inhibition of downstream molecules such as cytokines, growth factor, interleukins, and nitric oxide regulated by NFkB has been extensively studied, identification of upstream signaling molecules is poorly understood. Contrary to these findings [9]. found that supplementation of curcumin in the diets of humans affect the expression of toll like receptors. Since curcumin has been shown to affect the expression of many genes, he first screened several groups of TLR 2 that may be involved in the recognition of invading pathogens in monocytes and neutrophils. The mRNA expression levels of TLRs in curcumin treated human monocytic THP-1cells and neutrophilic-differentiated HL-60 cells were analyzed using semi-quantitative RT-PCR in this screening. Significant changes were seen only in mRNA level of TLR 2. Similarly [6]. reported that the relative mRNA expression levels of TLR 4 in the peripheral blood of the broilers were found to be increased ($P<0.05$) in the birds supplemented with graded levels of the garlic powder and holy basil leaf powder as compared to the untreated group. The antimicrobial action of essential oils in turmeric powder is attributed to phenolic in nature. They exert membrane damaging effects to microbial strains and stimulate leakage of cellular potassium which is responsible for a lethal action related to cytoplasmic membrane damage. Immunostimulant potential of turmeric powder is helpful in the treatment of immunosuppression [35-38]. It shows its immunomodulatory effect by increase in interferon-γ, interleukin-4, T-helper cells, NK cells thus reducing total bacterial count, increasing neutrophil and lymphocyte count and enhancing phagocytic activity and phagocytic index. Herbs can influence selectively the microorganism by an antimicrobial activity thus favours better nutrient utilization and absorption or the stimulation of the immune system. From the above reported studies and our result findings, it can be inferred that, supplementation of diet with turmeric powder improved performance, as holy basil leaf might have suppressed the growth of harmful organisms like E. coli, thereby creating a conducive environment for the growth of the beneficial microbes like *Lactobacillus sp.* and thereby, aid in digestion and give better performance. Medicinal herbs have shown to possess multiple immune modulatory actions like phagocytosis, modulation of immunoglobulin and cytokine secretion, cellular co-receptor expression, class switching, lymphocyte expression, and histamine release. In current work, it was observed that dietary inclusion of turmeric powder significantly enhances the relative mRNA expression of TLR 2 cell markers, which confirmed that these herbal feed additives could stimulate the T cell immune system in the plasma of layer birds.

Conclusions

The nutrigenomic expression analysis revealed that relative mRNA expression of TLR2 was found to be ($p<0.05$) increased in the treatment groups fed turmeric powder at all the four different levels. While, there was non-significant down regulation pattern of relative mRNA expression of TLR4 in the plasma of laying hens fed diet supplemented with turmeric powder at different levels. However, the data pertaining to the relative mRNA expression levels of TLR7 in the plasma of birds was non-significant in the different experimental groups. The treatments having turmeric powder in the layers' diet recorded potent immune modulating activity by showing significantly ($P<0.05$) up regulatory effect on relative mRNA expression of TLR2 and non-significant down regulation pattern of TLR4 in the laying hens. However, mRNA expression of TLR7 was not affected by dietary inclusion of turmeric powder.

Compliance with Ethical Standards

Statement on the Welfare of Animals

The animal experiment was conducted in accordance with guidelines approved by the Institutional Animal Ethics Committee, 12/CPCSEA Dated 6.2.2017 in the Department of Animal Nutrition, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar.

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