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Probiotic and prebiotic supplementation improving the production performance and immune characteristics of laying hens

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ABSTRACT

A 16 week feeding trial was conducted with 140 White Leghorn layers of 22-23±0.08 weeks of age which were distributed randomly into seven experimental groups, having 4 replicates with 5 birds in each. The laying hens of control group (T_1) were fed a basal diet (CP 18.04%), while in treatment groups T_2, T_3, T_4 basal diet was supplemented with probiotic (containing 5×10^8 cfu/g of *Lactobacillus fermentum*, 1×10^9 cfu/g of *Bacillus* spp., 1×10^9cfu/g of *Saccharomyces cerevisiae*) at levels of 0.5g, 1.0 g and 2.0 g /kg feed, respectively, while in T_5, T_6, and T_7 0.5g, 1.0g and 2.0g/kg feed of prebiotic (mannonoligosacchride of *Saccharomyces* cell wall-47g, formic acid-32g) was supplemented, respectively. The percent hen day egg production and feed efficiency (kg/dozen eggs and kg/kg egg mass) were improved ($P<0.05$) in probiotic and prebiotic incorporated diets. The mRNA expression levels of Toll like receptors (TLRs) TLR2 and TLR4 was increased ($P<0.05$) when graded levels of the probiotic and prebiotic were supplemented, while, TLR7 did not show any significant change. Thus, it can be inferred that the inclusion of the probiotic and prebiotic could enhance the production performance and immune status of birds by augmenting the T cell mediated immune response.

Key words: Gene expression, Layers, Prebiotic, Probiotic, Toll like receptors.

INTRODUCTION

There has been a recent surge in poultry sector for the complete removal of antibiotics from the animal and poultry production systems. Using antibiotics at sub-therapeutic lead to the development of drug-resistant bacteria due to antibiotic residues in animals and human (Cervantes, 2006). Thus, feed additives like probiotic and prebiotic have been searched which could replace the antibiotic substances by stimulating the growth of indigenous gastrointestinal microbiota thus protecting the host naturally (Tuohy *et al.*, 2005). A well balanced gut microbiota act as an efficient barrier against colonization of pathogens, produces metabolic substrates (e.g. vitamins and short-chain fatty acids) and in a non inflammatory manner stimulates immune system. In this perspective, probiotic and prebiotic could be possible solutions. Probiotic exert direct influence on pathogen elicited inflammatory response by their ability to down regulate specific signaling pathways like MAP kinase and NF-*kappa* beta (Yurong *et al*., 2005). Similarly, prebiotic could alter the gut microflora and immune system to reduce pathogenic colonization in stressed conditions (Gholap *et al*., 2009; Hajati and Rezaei, 2010). Relation between probiotic and immunobiotics has been proposed with reference to the microorganisms that stimulate activation of mucosal immunity in the gut associated lymphoid tissue (Clancy, 2003). Certain *in vitro* studies performed with TLRs (TLR) 2-transfected cells possibly to select specific immunobiotic lactic acid bacteria, has shown potential to stimulate the immune system (Tohno *et al*., 2007). In the present experiment, we have investigated whether the probiotic and prebiotic product, in compliance with maintaining production performance could enhance the relative mRNA expression of TLRs (TLR 2, TLR 4 and TLR 7) and thus improve immune status of laying hens by modulating immune response.

MATERIALS AND METHODS

The animal experiment was conducted in accordance with guidelines approved by the Institutional Animal Ethics Committee (IAEC), 12/CPCSEA Dated 8.4.2013 in the Department of Animal Genetics and Breeding, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar.

Birds, experiment design, and management: A total of 140 White Leghorn layers of 22-23±0.08 weeks, were randomly allotted to 7 treatments for 16 weeks. Each treatment consisted of 4 replicate pens with 5 birds in each. The laying hens of control group (T_1) were fed a basal diet (CP 18.04%) formulated as per BIS (Bureau of Indian Standards, 2007) standards as shown in Table 1. Experimental design is presented in Table 2. Seven dietary treatments included the basal diet (T_1) and the basal diets supplemented with 0.5g, 1.0g and 2.0g of the commercial grade probiotic product

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Table 1: Ingredients (%) and chemical composition (% DM basis) of basal diet.

Feed ingredients	Percent (%)
Maize	50.0
Groundnut cake	7.0
Soybean meal	13.0
Deoilled rice polish	12.0
Rice Polish	5.0
Fish Meal	6.0
Mineral Mixture	3.0
Salt	0.5
Shell Grit	3.5
Feed additive	*
Chemical composition	**(%) DM basis**
CP	18.04
CF	4.34
EE	3.61
Ash	7.80
Metabolizable energy (Kcal/Kg)**	2697.17

***Spectromix**-10g/ quintal , (Each g contained vitamin A- 82,500 IU, vitamin D_3-12,000 IU, vitamin B2- 50mg, and vitamin K- 10m) **Spectrimix-BE**-10g/ quintal, (Each g contained vitamin B1- 80mg, vitamin B_6 -16mg, Niacin- 120mg, vitamin B_{12}- 80mg, Calcium Pantothenate- 80mg, vitamin E -160mg, L-lysine HCl- 10mg, DL-Methionine -10mg, and Calcium- 260mg)

****** Calculated value as per Panda *et al*., 2002

(composed of 5×10^8 cfu/g of *Lactobacillus fermentum* , 1×10^9 cfu/g of *Bacillus spp*. and 1×10^9 cfu/g of *Saccharomyces cerevisiae*) in treatment groups T_2, T_3 and T_4, respectively. Whereas, the birds in the treatment groups T_5, T_6 and T_7 were fed 0.5g, 1.0g and 2.0g of the commercially available prebiotic product [mannonoligosacchride of *Saccharomyces* cell wall-47g, formic acid-32g, hydrated sodium calcium aluminosilicate (HSCAS)-upto 1 kg]. The populations of *Lactobacillus* and yeast were measured (Lei *et al*., 2009) in the feed samples of each group to check mixing status and probiotic viability in the feed. For each treatment group, feed intake was measured on weekly basis, whereas egg production data were recorded daily. Samples of 4 eggs from each treatment (1 egg per replication) at weekly intervals were randomly collected for measurement of egg weight, and egg mass production. Feed conversion ratio (FCR) was expressed as kilogram of feed consumed per dozen egg produced and per kg of egg mass produced. About 2 ml of blood was collected from each bird via brachial wing vein puncture 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. Total RNA extracted was dissolved in 30µl 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 (fermentas). The RT–PCR cyclic conditions were as: annealing at 25°C for 5 min, reverse transcription at 42°C for 1 hour, and deactivation at 70°C for 5 min in thermal cycler (Applied Bio system thermocycler). The cDNA was stored at –20°C till further use.

Real time PCR: For the analysis of temporal expression profile of different genes, real time PCR was carried out using Step I plus (ABI) Real Time PCR system. SYBR Green dye based PCR mastermix (Affymetrix) was used and all the instructions were followed as per the manufacter's. The reaction for the TLRs (TLR2, TLR4 and TLR7), and the endogenous control, β-actin gene was carried out in triplicate along with NTC (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 contain 2X SYBR Green PCR mastermix (Affymetrix) 12.5 µL), primers (forward and reverse 0.3M each), NFW (variable) and template (2 µL). Amplification was conducted with denaturation for 15 min at 95°C, followed by 40 cycles of denaturation for 5s at 95°C, and annealing/ elongation for 30s at 60°C, and a final melting curve analysis. The set of primers used for the real time PCR are as shown in Table 5.

Relative quantification by comparative C_t method ($\Delta\Delta C_t$ Method): The average C_t (Threshold cycle) value obtained for the TLRs 2, 4 and 7 (target) gene was normalized to β-actin (endogenous control). The data obtained was subjected to comparative C_t method (Livak and Schmittgen, 2001) for the analysis of the expression levels of targeted TLR gene and an endogenous control. The sample at 26 hour of incubation was selected as calibrator.

Sequencing of product: Amplicons were sequenced using with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) on an automatic ABI 3130

Table 2: Experimental design.

TreatmentGroups	Particulars	Number of replicates	Number of birds/ replicate	Total
T_1	Control-standard layer diet as per BIS (2007) specifications	4	5	20
T_2	Control+0.5g probiotic/Kg of feed	4	5	20
T_3	Control+1.0g probiotic/Kg of feed	4	5	20
T_4	Control+2.0g probiotic/Kg of feed	4	5	20
T_5	Control+0.5g prebiotic/Kg of feed	4	5	20
T_6	Control+1.0g prebiotic/Kg of feed	4	5	20
T_7	Control+2.0g prebiotic/Kg of feed	4	5	20
			Total	**140**

xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The sequence obtained shows 100% identity with the TLR sequence of chicken available in the global database.

Statistical analysis: Data were analyzed by one-way ANOVA as a completely randomized design using the GLM procedure of SAS Institute (SPSS, 2012). Differences among means were tested by the least significant difference method, and ($P < 0.05$) was considered to be statistically significant.

RESULTS AND DISCUSSION

Production performance: In layers feed intake was not affected by dietary inclusion probiotic and prebiotic in comparison to control. While, considerable (P<0.05) improvements in the hen day egg production values were observed in the hens supplemented with graded levels of probiotic and prebiotic. Thus, supplementation with highest (2g /kg) level of the probiotic and prebiotic in treatment groups T_4 and T_7 resulted in an increased advantage of 11.81% and 11.09% , respectively in hen day egg production (Table 4). Most of the studies conducted with laying hens have pointed out the positive relationship between probiotics, prebiotic supplementation and egg production rate (Stanley *et al*., 2000; Dowarah *et al*., 2016).

The dietary inclusion of probiotic and prebiotic (P<0.05) reduced feed intake (kg) for per dozen egg production and per kg egg mass production in T_4, followed by T_7 as compared to control (Table 3). As depicted in Table 4, highest percentage of egg mass production was observed in hens supplemented with probiotic and prebiotic @ 2g/Kg of feed in treatment groups T_4 and T_7, respectively. It was therefore hypothesized that there was strong correlation between increased nutrient utilization with probiotic supplementation in layer hens might be responsible for improved FCR (Swain *et al*., 2016). Improvements in FCR of 4.41, 8.82, 3.83 and 6.86%, was observed in treatments T_3, T_4, T_6 and T_7, respectively, compared to control (Table 3). These findings clearly indicate that higher levels (1g and

Table 3: Performance parameters *viz.* feed intake (g/hen/day), feed intake (Kg) per dozen of egg production and per egg mass production in layers under different dietary treatments.

Treatments	Feed Intake (g/hen/day)	Feed intake (Kg) per dozen of egg production	Feed intake (Kg) per egg mass production
T_1	117.34^{a}±1.61	2.04^{c}±0.03	3.13^{c}±0.05
T_2	118.29^{ab}±1.76	2.04^{c}±0.03	3.18^{c}±0.04
T_3	121.66^{ab}±2.04	1.95^{b}±0.02	3.01^{b}±0.03
T_4	120.38^{ab}±2.36	1.86^{a}±0.02	2.82^{a}±0.03
T_5	120.38^{ab}±2.4	2.03^{c}±0.03	3.15^{c}±0.03
T_6	121.38^{ab}±2.45	1.95^{b}±0.02	3.01^{b}±0.03
T_7	122.03^{b}±2.25	1.90^{ab}±0.02	2.90^{ab}±0.02

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

Table 4: Egg weight (g), Hen day egg production (percent) and Egg mass production (g/day/hen) in layers under different dietary levels of probiotic and prebiotic supplementation.

Treatments	Egg weight (g)	Hen day egg production (percent)	Egg mass production (g/day/hen)
T_1	53.63^{ab}±0.18	70.14^{a}±0.55	37.64^{a}±0.36
T_2	53.60^{ab}±0.28	69.73^{a}±0.75	37.40^{a}±0.45
T_3	53.95^{bc}±0.22	75.03^{c}±0.49	40.44^{b}±0.34
T_4	54.58^{c}±0.22	78.43^{d}±0.48	42.70^{c}±0.34
T_5	53.10^{a}±0.34	72.15^{b}±0.55	38.25^{a}±0.36
T_6	53.74^{ab}±0.27	75.40^{c}±0.61	40.43^{b}±0.42
T_7	54.22^{bc}±0.25	77.92^{d}±0.49	42.11^{c}±0.35

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

Table 5: Oligonucleotide sequences of sense and antisense primers for real-time PCR products 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.

2g/Kg of feed) of the probiotic and prebiotic used in the present study led to more efficient conversion of feed to egg mass.

Besides increase in egg weight, hens yielded eggs with 2.81 g, 5.07 g, 2.80 g and 4.48 g higher mass, in treatment groups T_3, T_4, T_6 and T_7 respectively, than control (Table 4). In association with inclusion of probiotic and prebiotic preparations there was output of eggs with increased weights and size in proportion to the increased egg mass.

Gene expression of Toll Like receptors (TLRs): In the present study, the differential mRNA expression of TLRs *viz.* TLR2, TLR4 and TLR7 gene transcripts in the blood of layers was determined by relative quantification method (ΔΔCT method) as depicted in Fig. 1,2 and Table 6. The nutrigenomic analysis revealed that layers fed diets supplemented with graded levels of 0.5, 1.0 and 2.0g/Kg of probiotic and prebiotic had ($P<0.05$) higher mRNA expression of TLR2 and TLR4 in the blood of layers as compared to control, while, no significant differences were observed in TLR7 mRNA expression (Fig. 1). Various strains of *Lactobacillus* could beneficially stimulate the T cell immune system in neonatal chicks, without affecting the B cell-mediated immune response (Sato *et al*., 2009). Similarly, dietary inclusion of probiotic and prebiotic alter the intestinal microbes and immune system to reduce colonization by the pathogens (Hajati *et al*., 2014), which explain the stimulation of the T cell immune system in the present study.

The data on nutrigenomic analysis depicted in Fig. 1 and Table 6 clearly indicated that supplementation of hens with highest level i.e. 2g/Kg of feed of either of the probiotic or prebiotic in treatment groups T_4 and T_7 had ($P<0.05$) higher mRNA expression of TLR2 and TLR4 in the blood, respectively, followed by hens fed with lower levels (1g/Kg of feed) of these additives in supplemental groups T_3 and T_6, while, it was least increased in the treatment groups T_2 and T_5. However, treatment groups T_4, T_7 and T_3, T_6 did not show any significant ($P<0.05$) differences among themselves. TLR2 recognizes gram positive bacteria by their lipoteichoic acid and yeast's zymosans (Uematsu and Akira, 2008). Zymosan present in the cell wall of *Saccharomyces cerevisiae*, having

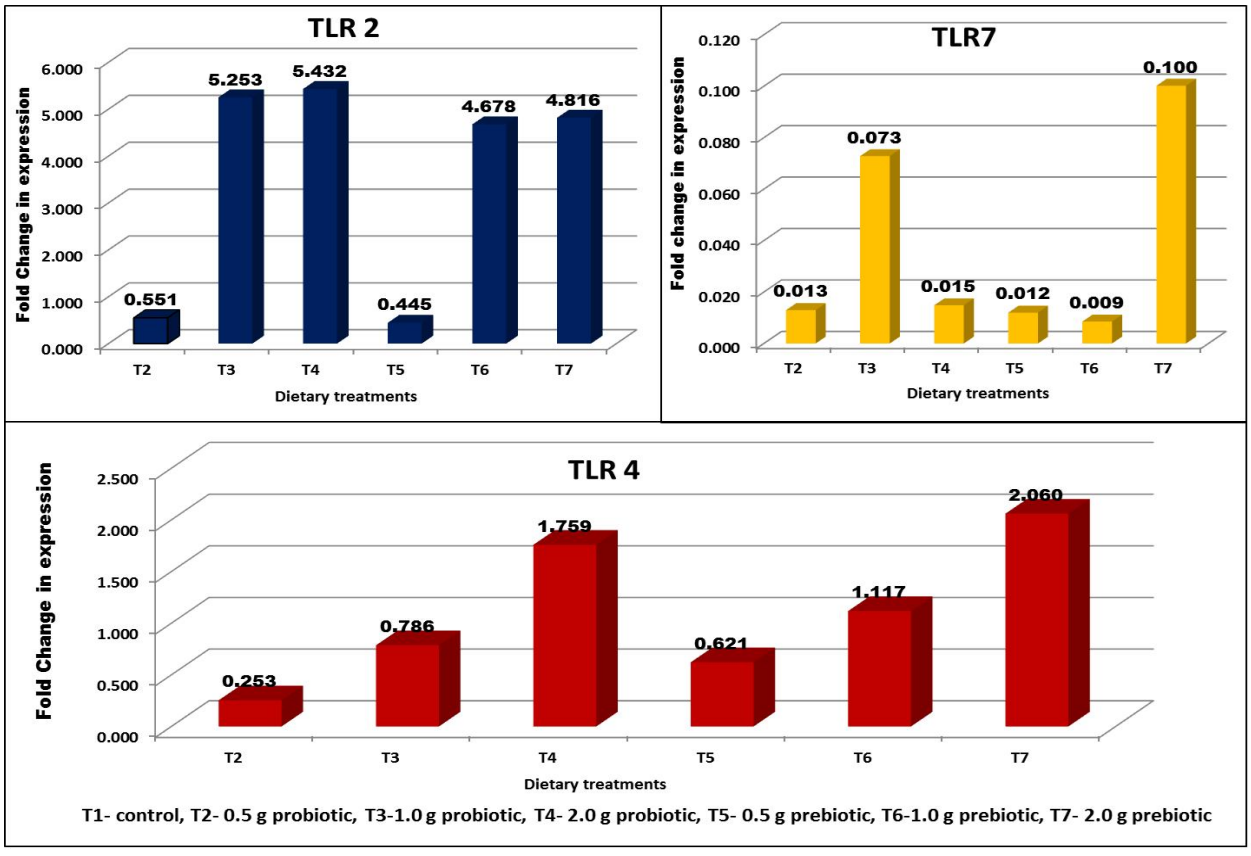


Fig 1: Effect of different dietary levels of probiotic and prebiotic supplemented diet on TLR 2, 4 and 7 mRNA expression. TLR2 and TLR4 mRNA expression is highest ($p<0.05$) in hens supplemented @ 1.0g and 2.0 g / Kg of the probiotic followed by the hens supplemented with highest (2.0 g) of the prebiotic. While, no significant ($p>0.05$) difference was observed on the expression of the TLR 7 mRNA levels among the different probiotic and prebiotic supplemented treatment groups as compared to control group.

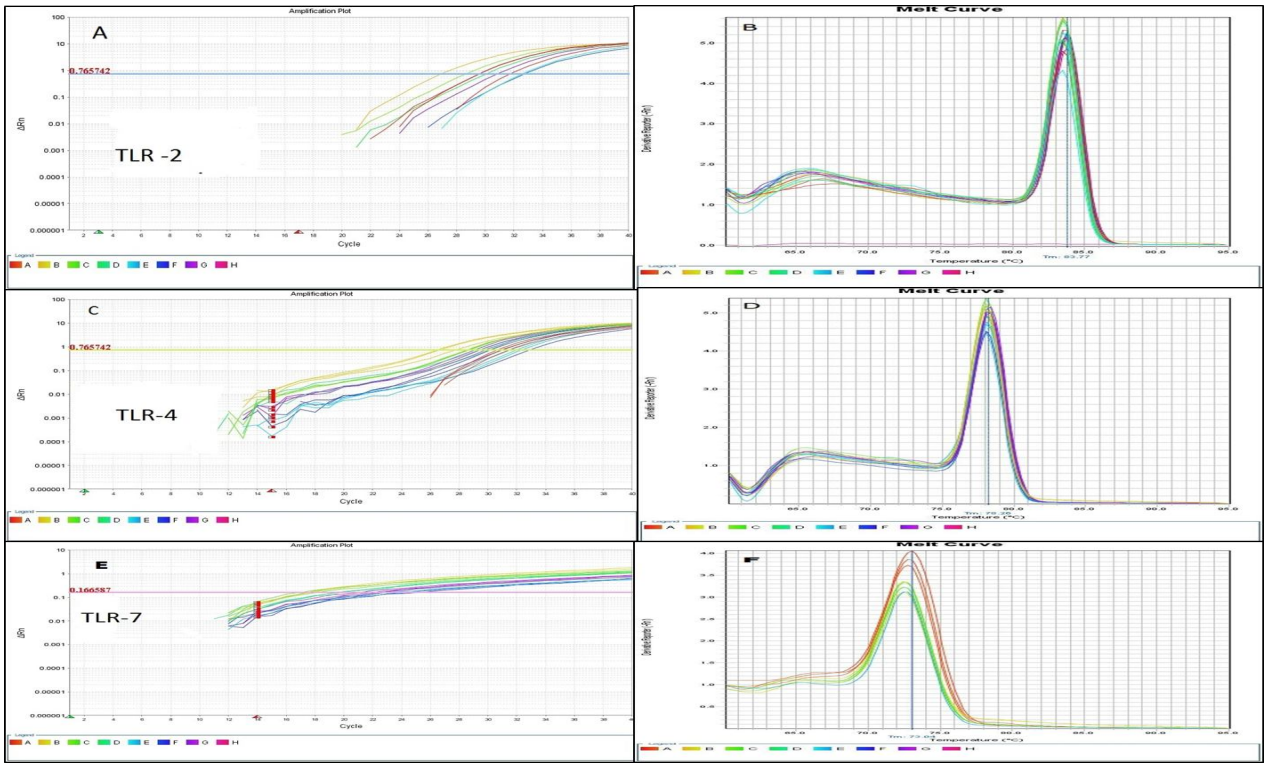


Fig 2: Amplification plot and melt curve for chicken TLR 2, TLR 4 and TLR 7.
Panel A and B represents amplification plot and melt curve for chicken TLRs 2.
Panel C and D represents amplification plot and melt curve for chicken TLRs 4.
Panel E and F represents amplification plot and melt curve for chicken TLRs 7.

Table 6: Relative quantitation expression analysis of the Toll like receptors (TLR 2, TLR 4 and TLR 7) with reference to the endogenous reference gene B actin

Sample Name	Target Name	C_T Mean	C_T SD	ΔC_T Mean	ΔC_T SE	$\Delta\Delta C_T$	RQ
T_1	**TLR 2**	31.45[a]	0.45	0.32	0.95	0.00	1.00
T_2		31.90[a]	1.42	1.18	0.83	0.86	0.55
T_3		27.99[b]	0.54	-1.76	0.50	-2.39	5.25
T_4		30.01[ab]	0.41	-2.12	0.62	-2.44	5.43
T_5		31.74[a]	2.30	1.49	1.42	1.17	0.44
T_6		25.32[c]	1.90	-1.62	1.24	-2.23	4.68
T_7		27.80[b]	0.70	-1.94	0.56	-2.27	4.82
T_1	**TLR 4**	31.76[ab]	1.22	0.63	1.15	0.00	1.00
T_2		33.38[a]	1.09	2.61	0.71	1.98	0.25
T_3		31.23[bc]	1.08	0.98	0.81	0.35	0.79
T_4		29.73[cd]	0.56	-0.18	0.49	-0.81	1.76
T_5		32.04[ab]	2.02	1.32	1.17	0.69	0.62
T_6		32.60[ab]	0.50	0.47	0.64	-0.16	1.12
T_7		29.19[d]	0.09	-0.72	0.38	-1.04	2.06
T_1	**TLR 7**	34.58	1.42	-0.93	2.26	0.00	1.00
T_2		36.12	0.21	5.80	1.03	6.21	0.01
T_3		33.22	2.66	3.30	1.58	3.78	0.07
T_4		35.85	0.56	5.60	0.65	6.07	0.01
T_5		36.68	3.26	5.91	1.91	6.38	0.01
T_6		37.12	0.34	6.40	0.24	6.87	0.01
T_7		34.97	3.25	2.84	1.96	3.32	0.10

[a, b, c, d, e, f] Mean values bearing different superscripts in a column differ significantly ($P<0.05$)

mannans as a major component, are chiefly identified by TLR 2, leading to the production of pro-inflammatory cytokines (Sato *et al*., 2003). Also, the diet incorporating of lactic acid species have potential to improve the gut mRNA levels of TLR 2. Thus, it can be inferred that relative mRNA expression of TLR2 got significantly increased by two - five fold in the blood of layers fed the probiotic and prebiotic supplemental diets which resulted from enhanced populations of *Lactobacillus fermentum* and yeast cell wall derivatives zymosans.

The TLR4 act as key receptor for lipopolysaccharide of gram negative bacteria, which is the integral component of their outer membrane complex (Higgins *et al*., 2010). Although, there might be down regulation in the relative mRNA expression levels of TLR4 in groups of birds fed the probiotic-added diet because probiotic inclusion in the diet of birds reduced the number of gram-negative bacteria such as *Coliform* (Lei *et al*., 2009). However, the mannans derived from the *Saccharomyces cerevisiae* cell wall could stimulate lipopolysaccharides which upregulates TLR4 expression in chicken cerebellum (Ansari *et al*., 2015). Cell wall products might have a co-stimulatory role on the induction of the systemic immune response (Erickson *et al*.,2000). This explains that relative mRNA expression of TLR4 was significantly increased upto two fold in layer's blood in the present study.

TLR7 recognizes some of the antiviral compounds and single-stranded viral RNA. In our study, no significant change in relative mRNA expression of TLR7 in hens fed with probiotic and prebiotic was found (Fig. 1 and Table 6), which indicated that these feed additives had no effect in modifying the T cell immune response to the viruses in the blood of layers. Based on production performance data and TLR2 mRNA gene expression it can be inferred that probiotic given @ 2g/Kg of feed showed better results as compared to the prebiotic given @ 2g/ Kg of the feed. Thus, probiotic and prebiotic supplemented diets increased the T cell subpopulation and enhanced the relative mRNA expression of TLR2 and TLR4, but not TLR7, in the blood of layers. Similarly, the egg production and feed efficiency in layers fed the probiotic and prebiotic supplemented diets was ($p<0.05$) better than birds fed basal diets.

CONCLUSION

Supplementation of probiotic and prebiotic at 2g/ Kg in laying diets increased the mRNA expression of TLR 2 and TLR 4 without any effect on TLR 7. Probiotic fed @ 2g/ Kg of diet was found to have slightly better expression and production enhancing profile than prebiotic. Thus, the inclusion of the probiotic and prebiotic could enhance the production performance and immune status of birds by augmenting the T cell mediated immune response and thereby protecting them from disease without decreasing productive traits as a possible substitution of antibiotics.

COMPETING INTERESTS

The authors have no conflicts of interest.

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