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Expression analysis of immunity related genes in White Leghorn layers supplemented with probiotics and prebiotics

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Abstract
One hundred and forty White Leghorn layers of 22-23±0.08 weeks of age were divided randomly into seven experimental groups, having 4 replicates with 5 birds in each. Feeding trial was conducted for period of 16 weeks. During the trial, in (T_1) control group layers were fed basal diet formulated as per BIS (2007) standards. While the basal diet was supplemented with probiotics (containing 5×10^8cfu/g of *Lactobacillus fermentum*, 1×10^9cfu/g of *Bacillus spp*. and 1×10^9cfu/g of *Saccharomyces cerevisiae*) @ 0.5g, 1.0g and 2.0g Kg^{-1} feed in T_2, T_3 and T_4 treatment groups, respectively. In the present study the relative mRNA expression levels of immunity related genes viz. Toll like receptors (TLRs) TLR 5, TLR 21 and TNF receptor associated factor 6 (TRAF 6) was analyzed in reference to endogenous reference gene β-Actin. The study results revealed that there was significant (P<0.05) downregulation in the relative mRNA expression levels of TLR 5 and its downstream gene TRAF 6 when increasing levels of the probiotics and prebiotics were supplemented to the laying hens. While, significant upregulation was observed in differential expression of TLR 21 due dietary inclusion of these feed additives. Thus, it can be concluded that the dietary inclusion of the probiotics and prebiotics can enhance the T cell mediated innate response in hens and in turn boost their immunity by significantly affecting the genes related immunity.

Keywords: Immunity, TRAF 6, prebiotics, probiotics, toll like receptors

Introduction
All over the world nowadays there is growing concern among the consumers about the ban on the use of antibiotics in poultry products [20]. Consequently, researchers are now looking towards viable alternatives for disease prevention and performance enhancing supplements. Thus, among the various feed additives searched out probiotics and prebiotics stand as suitable replacement candidates to antibiotics. Probiotics are the direct feed microbials which contain naturally occurring microbes including bacteria, fungi and yeasts. While prebiotics are defined as that can be utilized by intestinal microflora, which beneficially affect the host [5]. Probiotics and prebiotics attach to the gut mucosal receptors which are necessary for the attachment and colonisation of the pathogenic microorganisms and thus stimulates the immunity [7, 15]. Whenever, any pathogen invade the host's immune system it gets recognized by the family of Pattern Recognition Receptor (PRR) system which also includes Toll like receptors (TLRs). These TLRs augment the innate immune system by detecting a series of conserved molecular structures known as pathogen associated molecular patterns (PAMPs) present on pathogens. TLRs induce the production of reactive oxygen and nitrogen intermediates (ROI and RNI), inflammatory cytokines subsequently initiating the adaptive immunity in chicken. TRAF 6 gene encodes for proteins which mediates the signaling not only from the members of the TNF (Tumor necrosis factor) receptor superfamily, but also from the members of the Toll/IL-1 family. 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 [21]. Thus, probiotics and prebiotics have multiple beneficial effects on gut health and performance by directly stimulating their innate immune response via toll like receptors and TNF receptor associated factor 6 (TRAF 6). Keeping all this in mind, the present study aims to explore the effects of the probiotic product incorporating *Lactobacillus fermentum*, *Bacillus spp.*, *Saccharomyces cerevisiae and* prebiotic product incorporating mannan oligosaccharide of *Saccharomyces* cell wall, on the relative mRNA expression of the immunity related genes viz. toll like receptors and its downstream gene TNF receptor associated factor 6 (TRAF 6) in the blood of the White Leghorn layers.

Material 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 [2]. 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 (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 [mannon oligosaccharide of *Saccharomyces* cell wall-47g, formic acid-32g, hydrated sodium calcium aluminosilicate (HSCAS)-upto 1 Kg]. At the end of the feeding trial 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 supplier. The reaction for the TLRs (TLR5, TLR21) and TRAF 6, 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 5, 21 and TRAF6*;* 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 1.

Table 1: The primer sequences of sense and antisense primers for real-time PCR products determined

Gene[1]	Primer	Primer sequence[2]	Accession No.	Product size
TLR 5	Forward	5′-TCACACGGCAATAGTAGCAAC-3′	NM001024586	241
	Reverse	5′-CCTGAACACATCCAAACATAA-3′		
TLR 21	Forward	5′-CAAGAAGCAGCGGGAGAAG-3′	NM001030558	131
	Reverse	5′-TCAGGATGCGGTTAAAGCG-3′		
TRAF6	Forward	5′-ATGGAAGCCAAGCCAGAGTT-3′	NC 006092	144
	Reverse	55′-ACAGCGCACCAGAAGGGTAT-3′		
β-Actin	Forward	5′-GAGAAATTGTGCGTGACATCA-3′	L08165	152
	Reverse	5′-CCTGAACCTCTCATTGCCA-3′		

[1]TLR 5 = Toll-like receptor 5; TLR 21 = Toll-like receptor 21; TRAF 6 = TNF receptor associated factor 6.
[2]Primers for Toll-like receptors and β-actin were described by Jie *et al*. [9] and Sato *et al*. [16], respectively.

Relative Quantification by Comparative C_T Method ($\Delta\Delta C_T$ Method)

The average C_T (Threshold cycle) value obtained for the TLRs 5, 21 and TRAF6 (target) gene was normalized to β-actin (endogenous control). The data obtained was subjected to comparative C_T method [13] 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 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 [19]. Differences among means were tested by the least significant difference method, and (P<0.05) was considered to be statistically significant.

Results and Discussion

In this study we investigated the effect of supplementation of probiotics and prebiotics on the relative mRNA expression of immunity related genes viz. TLR 5, TLR 21 and downstream gene of TLR 5 i.e. TRAF 6, in the blood of laying hens. The differential mRNA expression of immunity related target genes in the blood of laying hens was determined by comparative quantification method ($\Delta\Delta C_T$ method) given by Livak and Schmittgen [13]. The results of the nutrigenomic analysis including the melt curve and amplification plots of all TLRs and TRAF 6 are depicted in Fig. 1, 2 and Table 2. The study results revealed that laying hens fed diets supplemented with increasing levels of probiotics and prebiotics had (P<0.05) lower mRNA expression of TLR 5 and its downstream gene TRAF 6 as compared to control (Fig 1and 2). Significantly greatest decrease (0.316 reaction quotient) in relative mRNA expression of TLR 5 was

observed in treatment group T_4 in which birds were fed highest amount (2g/Kg of feed) of probiotic. It is then followed by significant decrease of 0.420 and 0.515 RQ in T_3 and T_2 treatment groups fed @ 0.5g and 1.0g/Kg of the feed, respectively (Table 2). Similarly, among the prebiotic treatment groups laying hens supplemented at the highest rate of 2g/kg of the feed in T_7 had maximum decrease in relative mRNA expression of TLR 5 of 0.566 RQ, followed by T_6 (0.749) and T_5 (0.764) treatment groups fed prebiotic @ 1g and 0.5g/kg of feed, respectively.

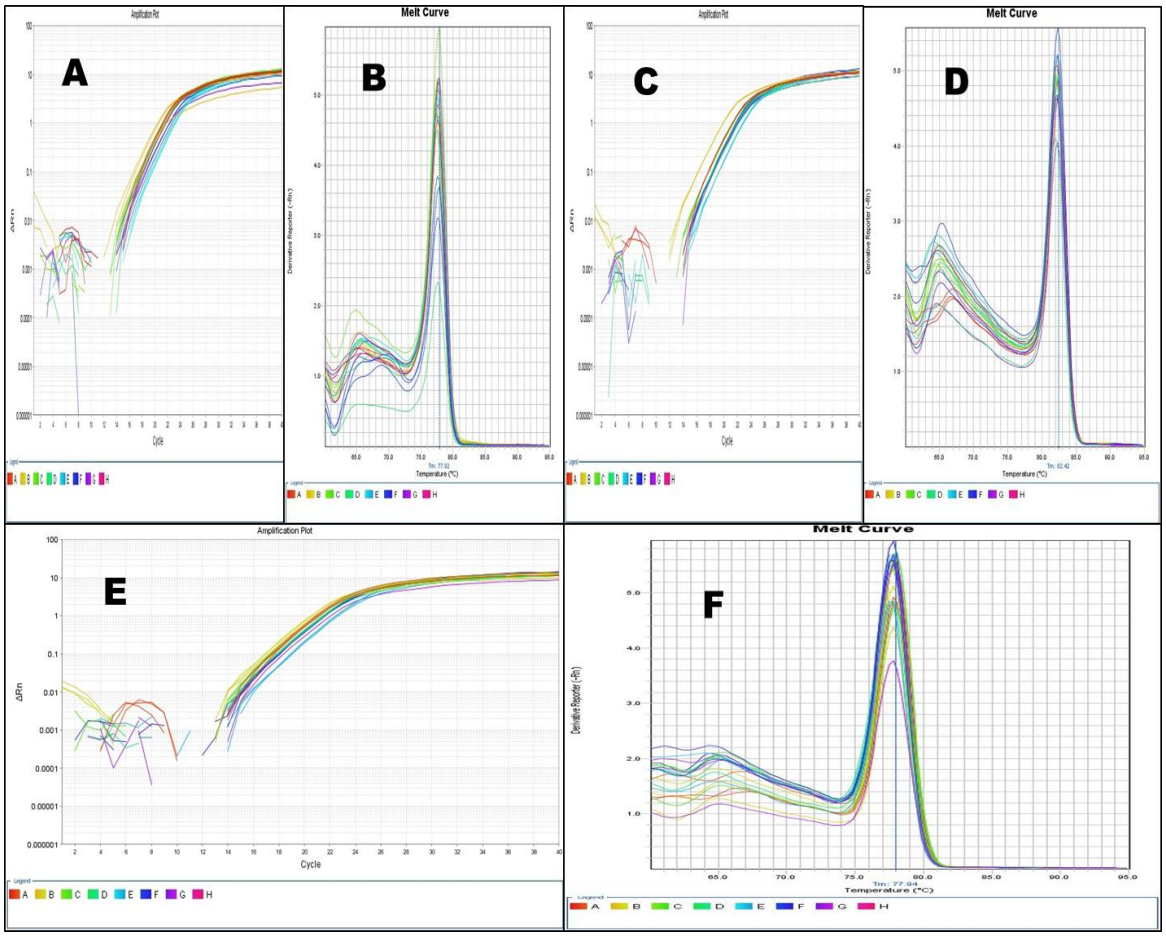

Fig 1: Melt curves and amplification plot for chicken TLRs 5, TLR 21 and TRAF 6.
Panel A and B represents amplification plot and melt curve for chicken TLR 5.
Panel C and D represents amplification plot and melt curve for chicken TLR 21.
Panel E and F represents amplification plot and melt curve for chicken TRAF 6.

Also, results in present study in Table 2 showed that similar trend of reduction was observed in relative mRNA expression of downstream gene of TLR 5 i.e. TRAF 6. TLR5 has been shown to recognize an evolutionarily conserved domain of flagellin through close physical interaction between TLR5 and flagellin [18]. Also, enforced expression of human TLR5 in CHO cells confers response to flagellin, a monomeric constituent of bacterial flagella [8]. As flagellin is found is found in the gram negative *E. coli* spp. and it is absent in the *Lactobacillus* spp. which might be responsible for the significant downregulation of the TLR 5 which recognizes bacterial flagellin in the present study. As probiotic-added to layers' diet in present study contain *Lactobacillus* spp. and *Saccharomyces cerevisiae*, these probiotic bacteria and yeast are known to reduce the number of pathogenic *Coliform* gram-negative bacteria such as *E. coli* [12]. As the population of gram positive beneficial microflora got increased due to addition of *Lactobacillus* spp. in probiotic product and a simultaneous decrease in harmful pathogenic gram negative *Coliforms* [4], this explains the reason for significant decrease in the relative mRNA expression of TLR 5 in the present study.

Similarly addition of prebiotic products like mannan oligosaccharides to chicken diet have shown to reduce colonization of pathogenic gram negative bacteria like *E. coli* and *Salmonella spp.* [1]. This explains the significant downregulation in TLR 5 expression due to supplementation of prebiotic product containing mannan oligosaccharide of *Saccharomyces* cell wall present in the current study. Individual TLRs initiate overlapping and distinct signaling pathways in various cell types such as macrophages, lamina propria and inflammatory monocytes. PAMP engagement induces conformational changes of TLRs that allow homo- or heterophilic interactions of TLRs and recruitment of adaptor proteins such as MyD88, TIRAP, TRIF, and TRAM. TLR5, which is highly expressed on the surface of these cells, uses MyD88 and activates NF-kB through IRAKs, TRAF6, TAK1, and IKK complex, resulting in induction of inflammatory cytokines. TLR5, which is expressed on the cell surface, signals through MyD88- IRAKs -TRAF 6- NF-kB to induce inflammatory cytokine productions [3]. As TRAF 6 is a downstream gene of TLR 5 so a decrease in expression of TLR 5 is obviously reflected in the concurrent downregulation of TRAF 6 gene, this explains the significant decrease in relative mRNA expression of downstream gene of TLR 5 i.e. TRAF 6 in present investigation.

Table 2: Relative quantitation expression analysis of the Toll like receptors (TLR 5, TLR 21) and TRAF 6 with reference to the endogenous reference gene β actin

Sample Name	Target Name	CT Mean	CT SD	ΔCT Mean	ΔCT SE	RQ
T 1	TLR 5	20.017^{bc}	0.281	4.398	0.000	1
T 2		20.465^{c}	0.269	5.355	0.958	0.515
T 3		20.998^{d}	0.210	5.650	1.253	0.420
T 4		21.701^{e}	0.152	6.059	1.661	0.316
T 5		19.309^{a}	0.520	4.786	0.389	0.764
T 6		19.880^{b}	0.150	4.815	0.417	0.749
T 7		20.122^{bc}	0.137	5.218	0.821	0.566
T 1	TLR 21	20.478^{c}	0.124	2.051	0.000	1
T 2		20.398^{c}	0.138	1.702	-0.348	1.273
T 3		19.185^{a}	0.035	1.655	-0.396	1.315
T4		20.500^{c}	0.069	1.214	-0.836	1.786
T 5		20.764^{d}	0.008	2.402	0.351	0.784
T6		21.298^{e}	0.090	1.784	-0.266	1.203
T 7		19.773^{b}	0.129	1.739	-0.312	1.241
T 1	TRAF 6	20.494^{b}	0.016	4.875	0.000	1
T 2		21.220^{c}	0.109	6.111	1.236	0.424
T 3		21.223^{c}	0.160	6.320	1.446	0.367
T4		22.093^{d}	0.029	6.451	1.576	0.335
T 5		19.117^{a}	0.009	4.594	-0.281	1.215
T6		20.641^{b}	0.016	5.576	0.701	0.615
T 7		21.133^{c}	0.136	5.785	0.911	0.532

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

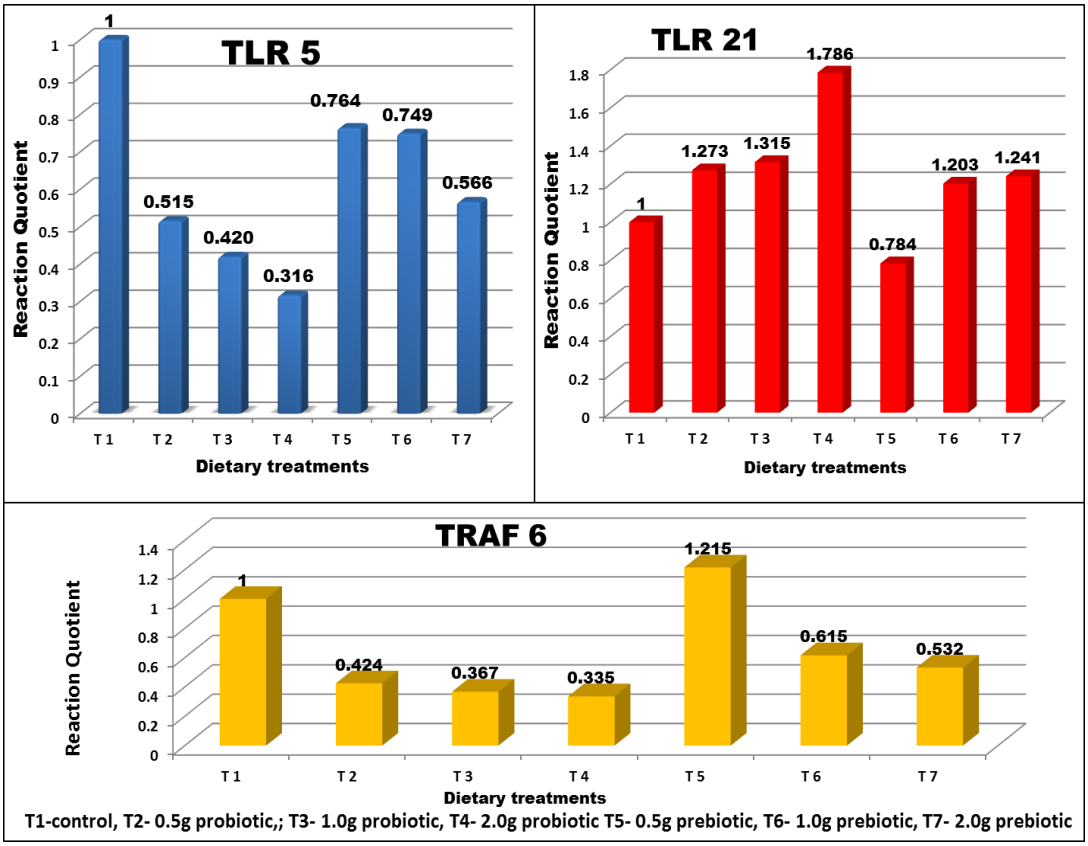

Fig 2: Differential mRNA expression analysis of genes related to immunity viz. TLR 5, TLR 21 and TRAF 6 due to dietary supplementation of probiotic and prebiotic in diet of White Leghorn layers.

The work carried out in this study depicted in Fig. 2 revealed that among the probiotic treatment groups, T_4 hens showed significantly (P<0.05) maximum increase (1.786 reaction quotient) in relative mRNA expression of TLR21 fed highest amount (2g/Kg of feed) of probiotic. It is then followed by significant increase of 1.315 and 1.273 RQ in T_3 and T_2 treatment groups fed @ 0.5g and 1.0g/Kg of probiotic, respectively. Similarly, among the prebiotic treatment groups laying hens supplemented at the highest rate of 2g/kg of the feed in T_7 observed highest (P<0.05) increase in relative mRNA expression of TLR21 of 1.241 RQ, followed by T_6 (1.203) and T_5 (0.784) treatment groups fed prebiotic @ 1g and 0.5g/kg of feed, respectively (Table 2). The innate immune response is a universal mechanism of host defense against infectious pathogens. TLRs functions on the basis of special receptors called PRRs (pattern-recognition receptors) which recognize conserved microbial structures called PAMPs (pathogen-associated molecular patterns). One

compound which has high stimulatory potential for innate immune response is bacterial DNA. Bacterial DNA contains unmethylated CpG motifs, which confer its immunostimulatory activity. TLR 21 is a nucleotide receptor that senses and responds to bacterial DNA (CpG motifs), which is analogous to the mammalian TLR 9 [11]. The frequency of potentially immunostimulatory CpG motifs is linearly dependent on the genomic G+C content of bacteria. Thereby, the overall load of CpG motifs in the intestine is dependent on the species assembly of microbiota and the cell numbers of particular species. Interestingly, Kant *et al.* [10] found that a number of species that are marketed as probiotics, *Bifidobacterium* sp., *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, had high counts and a high frequency of PuPu- CGPyPy and/or GTCGTT motifs. It has been suggested that the high frequency of CpG motifs in the DNA of *Bifidobacteria* may contribute to their reported beneficial effects via TLR9 stimulation [14, 17]. So in our study probiotic product containing *Lactobacillus* and *Bacillus* spp. might have played a significant role in upregulating the expression of TLR 21 via increased TLR 21 signalling. Thus, dietary inclusion of probiotic and prebiotic alter the intestinal microbes and immune system to reduce colonization by the pathogens [6], which explain the stimulation of the T cell immune system in the present study.

Conclusion

Thus in nutshell the results of this study lead us to conclude that probiotic and prebiotic products containing beneficial microbial species are able to reduce the population of potentially harmful microbes. Thus, these feed additives helps in boosting the immunity of the host by upregulating the expression of their immunity related genes viz. TLR 21 which identifies bacterial DNA; and downregulating the expression of TLR 5 and its downstream gene TRAF 6 which recognizes the flagellin present in pathogenic gram negative bacterial species, whose number got decreased due to dietary supplementation of probiotics and prebiotics in laying hens.

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