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Supplementation of Ashwagandha root powder improving gut micro flora and immune response of broilers

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Abstract
A 6 week feeding trial was carried out to study the influence of dietary inclusion of Ashwagandha root powder on gut microflora and immune status of broiler birds. A total of 300, one day-old commercial broiler chicks were randomly distributed into six dietary treatments with five replicates per treatment and each replicate has ten birds. The control group (T_1) was offered maize- soybean meal based diet which was formulated as per specifications to fulfill the metabolizable energy (ME) and crude protein requirements of broilers. The first group was kept as a control (T_1) and given the basal diet without antibiotic while second (T_2) basal diet with antibiotic, third (T_3), fourth (T_4), fifth (T_5) and sixth (T_6) groups were supplemented with Ashwagandha root powder @ 0.25, 0.5, 0.75 and 1%, respectively in the diet. On day 42, feeding chicks Ashwagandha root powder at different levels significantly (P<0.05) reduced *Escherichia coli* (*E. coli*) count while *Lactobacillus* spp count were significantly higher (P<0.05) in the gut when compared to control group. The mRNA expression levels of Toll like receptors (TLRs) TLR2 was increased and a down regulation was observed in TLR4 (P<0.05) when different levels of Ashwagandha root powder were supplemented, while, TLR7 did not show any significant change. Supplemented diet with Ashwagandha root powder may be useful in modulating broilers gut by controlling enteropathogens that help to utilize feed efficiently, which subsequently enhances the growth performances of broiler chickens. Thus, it can be inferred that the inclusion of the Ashwagandha root powder could influence gut microbiota and immune status of birds by augmenting the T cell mediated immune response.

Keywords: Gut micro flora, gene expression, toll-like receptors, Ashwagandha

Introduction
The poultry industry in the country in present era has grown rapidly on account of its low capital investment, early assured returns, short generation intervals and limited land requirements (Singh *et al.,* 2014) [23]. Use of synthetic growth promoters like antibiotics has led to success in limiting most of the prevalent bacterial diseases which affected man and animals in epidemic proportions. At the same time, inadvertent and overuse of antibiotics resulted in disadvantages like high cost of production, toxicity and development of resistance and environmental and health hazards. To eliminate antibiotics, several studies has focused on the development of alternative strategies to maintain poultry health and enhance performance within intensive systems, and numerous substances, commonly known as natural growth promoters (NGPs) have been identified as effective alternatives to antibiotics. Commonly used growth promoters are prebiotics, probiotics, synbiotics, enzymes, acidifiers and phytobiotics. Phytobiotics are NGPs, which have been growing in popularity as feed additives, due to their beneficial effect on gut health and immunity and growth performance. Herbal preparations are widely used as feed additives for enhancing growth, reducing feed cost by improving feed conversion ratio and for building better immunity in broiler production (Biswas *et al.,* 2012 and Pandey *et al.,* 2013) [6, 15]. Furthermore, these herbal feed additives have no side effects on the health of birds and increase the performance of broiler by increasing live weight gain, feed conversion ratio (Samarth *et al.,* 2002) [18] and immunity (Bhardwaj *et al.,* 2011 and Kumari *et al.,* 2012) [4, 10]. India with its rich traditional heritage is well known for Ayurvedic medicine system due to its therapeutic potential, which has been now practiced for human as well as animal health care. *Withania somnifera* (Ashwagandha) holds a celebrated position in the Indian *materia medica*. Ashwagandha plant constitutes alkaloids and steroidal lactones, but the withanine, the main alkaloid found in its roots and leaves is thought to be responsible for its biological activity.

Other constituents include saponins containing an additional acyl group (sitoindoside VII and VIII). It consists of following properties viz. adaptogenic, antidepressant, liver-tonic, antioxidant, immune modulator, immune adjuvant (Ziauddin *et al.*, 1996) [27] and also improve feed intake, body weight gain, FCR, hematological profile and immunological status, neuro-protective and rejuvenate muscles (Ansari *et al*., 2008) [1]. 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 (Sheoran *et al.*, 2017b) [21]. Keeping in view the various medicinal properties of Ashwagandha and its beneficial effects on various physiological processes, the present study was planned to evaluate the effect on gut micro flora and immune system of broiler birds fed Ashwagandha supplemented diet.

Material and Methods

The animal experiment was conducted in accordance with guidelines approved by the Institutional Animal Ethics Committee (IAEC), 235/CPCSEA dated 1-8-2000 in the Department of Animal Nutrition, LUVAS. Three hundred, one day old broiler chicks, were purchased from a local commercial hatchery. The chicks were individually weighed, wing banded and randomly distributed into 30 subgroups means six dietary treatments with five replicates per treatment and each replicate has ten birds. Basal ration was formulated as per BIS (2007) [5] to fulfill the metabolizable energy (ME) and crude protein requirements of birds. Level of crude protein in starter (0-4weeks) and finisher (4-6weeks) Ration was 23 and 20%, respectively. The respective ME content was 3000 and 3200 KCal/kg are presented in Table 1. The first group was kept as a control (T_1) and given the basal diet without antibiotic while second (T_2) basal diet with antibiotic, third (T_3), fourth (T_4), fifth (T_5) and sixth (T_6) groups were supplemented with Ashwagandha root powder @ 0.25, 0.5, 0.75 and 1%, respectively in the diet. Birds were vaccinated against F_1 strain of Ranikhet disease on 3rd day and Infectious Bursal Disease on 14th day. The chicks were kept hygienically on floor litter system in separate pens. The chicks were brooded at 35ºC during the first week. The birds were vaccinated against prevailing diseases adopting a standard protocol. The fortnightly record of the feed intake and body weight gain was maintained for each replicate to calculate the feed efficiency per bird.

Table 1: Chemical composition of feed ingredients used in ration formulation

Ingredient	CP (%)	CF (%)	EE (%)	TA (%)	Lysine* (%)	Methionine* (%)	ME* (kcal/kg)
Maize	9.31	2.48	3.49	2.25	0.18	0.15	3300
Soyabean meal	45.40	3.96	3.16	8.47	2.57	0.76	2230
Fish meal	47.40	1.82	5.16	26.62	1.42	1.42	2210
Ashwagandha powder	2.91	6.34	2.30	4.41	-	-	245

*Calculated values (Singh and Panda, 1992) [22]

CP= Crude protein, CF= Crude fiber, EE=Ether extract, TA= Total ash, ME= Metabolizable energy

At the end of 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. For gut microflora, one bird from each replicate was randomly selected and the ileal contents were collected aseptically. Samples were weighed (1 g), transferred to sterile tubes and homogenized with sterile 0.9% normal saline solution (1:1). Then the solutions were mixed on vortex. Serial dilutions of samples were made up to five dilutions. From each dilution 0.1 ml was poured and spread uniformly on Mac Conkey lactose agar for *Escherichia coli* and on MRS (De Man, Rogosa and Sharpe agar) for *Lactobacilli*. *Escherichia coli* plates were incubated at 37 °C for 24 h and *Lactobacillus* for 24 to 72 h at same temperature. The microbial counts were determined as colony forming units (cfu) per gram of samples.

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 c-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 2.

Relative quantification 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 was subjected to comparative Ct method (Livak and Schmittgen, 2001) [11] 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 ABI3130xl 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) [24]. Differences among means were tested by the least significant difference method, and ($P < 0.05$) was considered to be statistically significant.

Table 2: 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) [19] and Bai *et al*. (2008) [3], respectively.

Results and Discussion

Gut Micro flora

Mean values of *E. coli* (log cfu/g) ranged from 2.89 (T_6) to 4.75 (T_1) among different dietary treatments are represented in Table 3. There was a significant reduction in *E. coli* count in T_5 and T_6 groups as compared with the control group (T_1). Total *Lactobacilli* (log cfu/g) ranged from 3.28 (T_2) to 5.24 (T_5) and highest value was recorded in the T_5 (5.24) group supplemented with 0.75% Ashwagandha root powder. It was significantly (P<0.05) higher than antibiotic supplemented group (T_2) as well as from control (T_1). Other treatment groups T_3, T_4 and T_6 were also significantly (P<0.05) higher as compared to control group. Report documented by Rizwana *et al.* (2012) [17] revealed that extracts of *Withania somnifera* especially methanolic leaf extract were more potent against MRSA (methicillin-resistant *Staphylococcus aureus*) than standard antibiotic, vancomycin (30µg) used in the study. They concluded that chloroform, acetone, methanolic and ethanolic extracts of *Withania somnifera* might be exploited as natural drug for treatment of several infectious diseases. Another study by Attia *et al.* (2017) [2] reported a positive (P<0.05) influence on cecal microflora count (decreased *coliforms* and increased lactobacilli count) by inclusion of the plant extract blend. However; intestinal histomorphological parameters were not significantly influenced. This lowered caecal *coliform* count could be attributed to the antibacterial properties of the utilized plant extract components in the plant extract blend or to increased nutrient digestibility and subsequently less undigested nutrients available for bacterial fermentation in the ceacum. Similarly, Kumar *et al.* (2018)[9] reported that birds supplemented with corn soyabean based diet supplemented with 100 mg *Withania somnifera* extract/kg diet reduced *E. coli* counts significantly (P<0.05) than other groups.

Table 3: *E. coli* (log cfu/g) and total *Lactobacilli* (log cfu/g) count of the ileal content of the experimental birds under different dietary treatments

Treatments	*E. coli* (log cfu/g)	*Lactobacilli* (log cfu/g)
T_1	4.75^{b}±0.06	3.58^{b}±0.17
T_2	3.81^{ab}±0.05	3.28^{a}±0.18
T_3	4.37^{ab}±0.32	4.65^{c}±0.07
T_4	3.79^{ab}±0.34	5.05^{d}±0.05
T_5	2.92^{a}±0.75	5.24^{d}±0.02
T_6	2.89^{a}±0.74	5.21^{d}±0.02

Means bearing different superscripts in a column differ significantly (P<0.05)

mRNA gene expression of TLRs

The differential expression level of TLRs, viz. TLR 2, TLR 4 and TLR 7 gene transcripts in the 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 result is said to be significant (P<0.05) when there is twofold change in RQ (reaction quotient) in comparison with control. The nutrigenomic expression analysis as presented in Table-4 and also depicted in Figure 1, revealed that relative mRNA expression of TLR 2 of broilers was found to be significantly (P<0.05) enhanced in the treatment groups T_5 and T_6 supplemented with 0.75% and 1.0% of the Ashwagandha root powder, respectively. While, as presented in Table 4 and shown in Figure 2 at the end of the 6 weeks of experimental period broilers had significant down regulation pattern of relative mRNA expression of TLR 4 in the plasma of broilers fed diet supplemented with 0.25%, 0.50%, 0.75% and 1.0% of Ashwagandha root powder in the treatment groups T_3, T_4, T_5 and T_6, respectively as compared to control (T_1) group. However, the data pertaining to the relative mRNA levels of TLR 7 shown in Table 4 and Figure 3, in the plasma of birds revealed non-significant differences in the experimental groups T_2, T_3, T_4 and T_5 and T_6 as compared to the control group (T_1). The experimental treatments containing Ashwagandha root powder in the broiler's diet had potent immune modulating activity by showing stimulatory effect on relative mRNA expression of TLR 2 and down regulation pattern of TLR 4 of the commercial broilers.

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

Sample Name	Target Name	C_T Mean	C_T SD	ΔC_T Mean	$\Delta\Delta C_T$	RQ
T_1	TLR 2	21.14	0.08	5.39	0	1
T_2		20.90	0.12	5.07	-0.32	1.25
T_3		20.81	0.14	5.02	-0.37	1.29
T_4		19.60	0.07	4.49	-0.90	1.87
T_5		19.00	0.11	3.93	-1.46	2.75
T_6		20.01	0.16	3.98	-1.41	2.64
T_1	TLR 4	20.44	0.09	4.69	0	1
T_2		21.29	0.15	5.46	0.77	0.58
T_3		21.12	0.12	5.33	0.64	0.64
T_4		20.56	0.05	5.45	0.76	0.59
T_5		20.65	0.13	5.58	0.89	0.54
T_6		21.62	0.07	5.59	0.90	0.53
T_1	TLR 7	27.092	0.17	11.35	0.0	1
T_2		27.156	0.19	11.33	-0.02	1.01
T_3		27.129	0.28	11.34	-0.00	1.00
T_4		26.443	0.38	11.34	-0.01	1.01
T_5		26.399	0.22	11.33	-0.01	1.01
T_6		27.343	0.16	11.32	-0.03	1.02
T_1	β actin	15.75	0.56	-		-
T_2		15.83	0.52	-		-
T_3		15.79	0.47	-		-
T_4		15.11	0.44	-		-
T_5		15.07	0.50	-		-
T_6		16.03	0.49	-		-

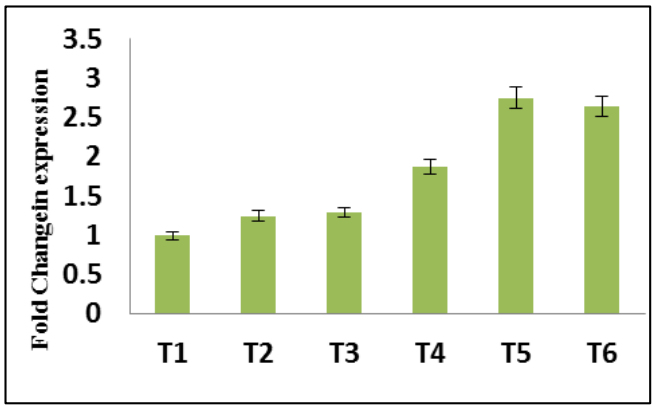

Fig 1: RQ of TLR 2 Effect of different dietary levels of Ashwagandha root powder on relative mRNA expression of TLR2

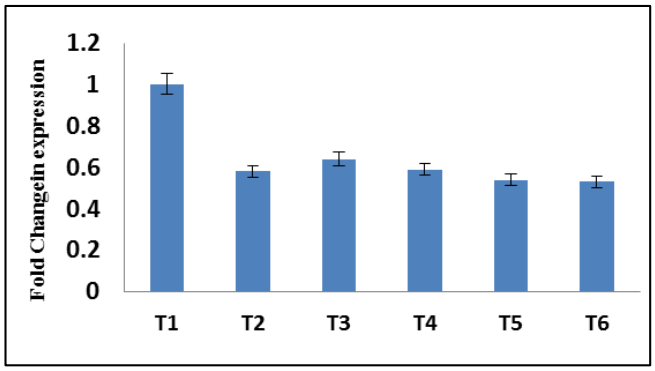

Fig 2: RQ of TLR 4 Effect of different dietary levels of Ashwagandha root powder on relative mRNA expression of TLR4

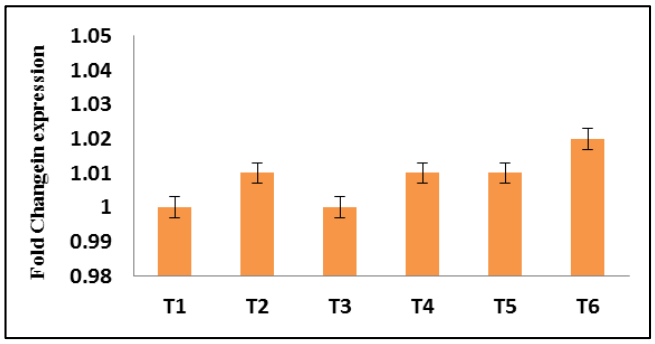

Fig 3: RQ of TLR 7 Effect of different dietary levels of Ashwagandha root powder on relative mRNA expression of TLR7

T1: Control, T2: Control with Antibiotic, T3: 0.25% Ashwagandha, T4: 0.50% Ashwagandha, T5: 0.75% Ashwagandha, T6: 1% Ashwagandha

Our study for TLR2 are in consonance with finding of Sheoran *et al.* (2017a) [20], who concluded that the addition of garlic powder and holy basil leaf powder at higher level of 1% of the feed either alone or in combination in the diet of the broilers increased the relative mRNA expression of TLR 2, 4 and 7. Our study in case of expression of TLR4, TLR 7 is in contrary to Sheoran *et al.* (2017a) [20]. Medicinal herbs has 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 (Mahima *et al*., 2012) [13]. In current work, it was observed that dietary inclusion of Ashwagandha root powder significantly modulate the relative mRNA expression of TLR cell markers; this confirmed that these herbal feed additives could stimulate the T cell immune system in the plasma of broiler birds. In the present investigation, we found that there was a significant increase in the relative mRNA expression of TLR 2 in the plasma of the broilers fed diet supplemented with 0.75% and 1% Ashwagandha root powder. TLR 2 recognizes a variety of microbial components. These include lipoproteins/ lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from Gram-positive bacteria (Takeda and Akira, 2005) [25]. Thus, it can be inferred that relative mRNA expression of TLR 2 got significantly increased by two fold in the blood of broiler fed the Ashwagandha supplemental diets which resulted from enhanced populations of *Lactobacillus fermentum* and yeast cell wall derivatives zymosans. TLR 4 is the principal receptor for lipopolysaccharide. This is a major component of outer membrane of gram-negative bacteria (Kannaki *et al*., 2010) [8]. Several studies have shown that the essential oils and biologically active compounds in herbs are effective against bacteria such as *E. coli, Shigella* spp. *Salmonella typhi*, and *Pseudomonas aeruginosa* (Prakash and Gupta, 2005) [16]. The antimicrobial actions of essential oils in herbs are due to phenolic compounds present in them. They exert membrane damaging effects to microbial strains and stimulate leakage of cellular potassium this is responsible for a lethal action related to cytoplasmic membrane damage (Mahamood, *et al*., 2008) [12]. They show its immune modulatory effect by increase in interferon-γ, interln eukin-4, T-helper cells, NK cells (Mondal *et al*., 2011) [14], 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 favors better nutrient utilization and absorption or the stimulation of the immune system (Wenk, 2003) [26]. From the above reported studies and our result findings, it can be inferred that, supplementation of diet with 0.75% Ashwagandha improved performance, suppressed the growth of harmful organisms like *Coliforms*, thereby creating a conducive environment for the growth of the beneficial microbes like *Lactobacillus*, *Bifidobacteria* spp. and thereby, aided in digestion and gave better performance. 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 Ashwagandha root powder did not significantly change the relative mRNA expression of TLR 7 in the plasma of the broiler birds, which indicated that Ashwagandha had no effect in modifying the T cell immune response to the viruses in the blood of broilers. In contradictory to our findings, Huang (2017) [7] revealed that there was a positive regulation of TLR4 mRNA expression in the liver, kidney, spleen, heart and small intestine of broiler chickens and that acute heat stress might cause organ injury via increased TLR 4 mediated inflammation; however, this finding need to be further investigated in broiler chickens. In addition, the TLR 4 mRNA expression was different among the organs of broilers submitted to 10 hours of heat stress (small intestine > heart > liver > spleen and kidney). In those submitted to two hours of heat stress, TLR 4 mRNA expression was significantly reduced in the spleen, but not in the liver, kidney, and heart, suggesting that the immune function plays a vital role at the beginning of acute heat stress and TLR signaling is highly physiologically relevant to the organs.

Conclusion

On the basis of above study it can be concluded that the supplementation of Ashwagandha root powder at higher levels 0.75% and 1% results in up regulation of TLR 2 and down regulation of TLR 4. This might be due to enhanced growth of beneficial gram positive bacteria (*Lactobacilli* spp) and decreased gram negative (*E. coli*) bacteria in birds.

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