G.J.A.S.R

GLOBAL JOURNAL OF ANIMAL SCIENTIFIC RESEARCH

Journal homepage: www.gjasr.com

Print ISSN:2345-4377 *Online ISSN:2345-4385*

Original Article

Effect of Feeding Thymolina Powder on the Immune System and Gene Expression Interleukin II

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ABSTRACT

Medicinal plants are beneficial to intestinal palatability and performance, have antimicrobial properties, have a wide range of antioxidant activities, stimulate the immune system, and promote nutritional absorption. 320 one-day-old Ross 308 broiler chicks were utilised in this study, which followed a completely randomised design with four treatments, four replicates, and each replicate contained 20 broiler chickens. Control, 0.5, 1, and 2% in the food of hens are among the experimental treatments. Thymolina is an antibacterial powder medication derived from the combination of four medicinal herbs (*Salvia officinalis, Matricaria chamomilla, Teucrium polium and Origanum majorana*). During the experiment period, chickens had free access to water and food and the livestock raising management was conducted in terms of lighting, humidity, ventilation, and vaccination in accordance with the guide requirements of Ross 308 broiler chickens. Weighting was conducted weekly and at the end of feed intake, weight gain and feed conversion ratio of chickens were

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Cite this Article: Hosseini, S.M., Chamani, M., Seidavi, A., Sadeghi, A. A., & Ansari-Pirsaraei, Z. (2021). Effect of Feeding Thymolina Powder on the Immune System and Gene Expression Interleukin II. *Global Journal of Animal Scientific Research,* 9(2), 160-170.
Retrieved from http://www.gjasr.com/index.php/GJASR/article/view/98

Article History: Received: 2021.09.15 Accepted: 2021.11.25

measured. To investigate the body's immune system of broiler chickens, the HI and SRBC tests were used. To do so, each replicate of a bird was selected and 1 ml per kg of live weight of chickens of sheep red blood cells (SRBC) was injected was injected into the pectoral muscle at the age of 28 years old. 7 days after the injection, blood sampling from the brachial vein of the same birds as 2 ml was conducted and blood serum was separated by centrifugation at 3000g for 10 min. the amount of antibody titers against SRBC in serum obtained using the HA method and concentration of Newcastle disease vaccine titers obtained by HI were determined. In addition, to the gene expression of interleukin II, the same birds were selected, and at the end of the period, the amount of 1-2 ml blood was taken under the wing of the venous vessel, and then the investigation of the amount of the gene expression of interleukin II was conducted using specific primers, GEN, and Real-Time PCR Reaction. Newcastle disease vaccine titers and SRBC were experimented with by experimental treatments and the alloy of anti-body titers increased. The gene expression of interleukin II increased and significant differences among different treatments were observed ($P<0.05$). Overall, the results showed that including Thymolina in the diet of broiler chickens improves their immune system by raising IL-2 gene expression. Therefore, it can be effective on the immune responses of broiler chickens and cause the improvement creation of the favorable and effective immune system in broiler chickens.

Keywords: Immune, Interleukin II, Gene Expression, Thymolina, Ross 308, Broiler.

INTRODUCTION

Medicinal plants, due to having suitable effects on intestinal palatability and performances (Jugl-Chizzola *et al.*, 2006), antibacterial effects (Özer *et al.,* 2007), wide antioxidant activities (Wei and Shibamoto, 2007), stimulation of the immune system (Roth-Maier *et al.,* 2009) and improvement in nutrient absorption (Windisch *et al.,* 2008) are added to diets of broiler chickens. Therefore, in the present study, an antibacterial medicinal plant called Thymolina in powder was used. In making this drug, 4 medicinal plants *Salvia officinalis, Matricaria chamomilla, Teucrium polium* and *Origanum majorana* were composed. This composition contains 1% active ingredient thymol. Chaturverdi *et al.*, (1997) reported that Sanguinarine increases the titration of antibodies against the SRBC.

Cytokines are proteins that are secreted from specific innate immune cells and are the mediators of a lot of their activities. The IL-2 is one of the most important cytokines of the body in regulating immune response which causes amplification and division of lymphocytes T as important parts in reactions related to the immune system.

In recent years, immune cytokines such as the chIL-2 are employed in the poultry industry. These cytokines can increase the efficacy of vaccines by amplification of

lymphocytes T, development of lymphocytes B, and activation of natural immune killer cells (NK).

Deng *et al.*, (2007) reported that supplementation of the diet of pigs is extracted from Chinese herb (Semen cassia) with polysaccharides results in the increase in the gene expression of IL-1β and IL-2 in blood mononuclear cells and lymph nodes as well as the expression of IL-1β jejunal mucosa.

In addition, the results of researches done by other researchers indicate that the use of Angelica Gigas (Sang, 1998), garlic (Munir, 2015), Ganoderma Lucidum (Wang *et al.*, 2002), Ginseng (Tan and Vanitha, 2004) and powder Astragalus (Xi *et al.*, 2014) causes the increase in the IL-2.

MATERIALS AND METHODS

Animals, Experimental Design and Procedure

In the present experiment, 320 one-day-old Ross 308 broiler chickens were used based on a completely randomized design with 4 treatments, 4 replicates, and each replicate is a pan containing 20 broiler chickens. Experimental treatments include basal diet containing no supplemented Thymolina (control), basal diet supplemented with 0.5% Thymolina, basal diet supplemented with 1% Thymolina and basal diet supplemented with 2% Thymolina in chickens' diet. Thymolina is an anti-bacterial powder drug that is made by composing 4 medicinal plants (*Salvia officinalis, Matricaria chamomilla, Teucrium polium,* and *Origanum majorana*) (Table 1).

Table 1: The chemical composition of Thymolina

Thymolina Ingredient	Important constituents%
Salvia Officinalis	camphor (37.17), 1,8 cineole (31.1), α -Thujone (20.34), β-thujene (3.37), borneol (2.02)
Matricaria Chamomilla	(*E*)- β -farnesene (24.19), guaiazulene (10.57), α -bisabolol oxide A (10.21), α -farnesene (8.7) and α -bisabolol (7.27)
Teucrium Polium	β –caryophyllene (29.5), farnesene-cis-b (11.2), β -pinene (5.2), carvacrol (8.3), bicyclogermacrene (6.4), β-pinene (5.2)
Origanum Majorana	Trans-Caryophillene (19.08), Gamma-Cadinene (10.91), Trans-Beta-Farnesene (8.65), Gamma-Terpinene (6.29), Apiol (5.62)

In the present experimental study, the corn, wheat- soybean meal-based diet was used to supply chickens' nutritional needs in different breeding periods (Starter (0-15 d), Grower (16-28 d), Finisher (29-42 d)), experimental diets were prepared and regulated based by the UFFDA software, and diets were formalized based on Ross 308 broiler chicken's nutritional requirements (Table 2).

Table 2: Ingredient composition of basal diet

Ingredient (g/Kg unless noted)	Starter (0-15 d)	Grower (16-28 d)	Finisher (29-42 d)
Yellow Corn	430.25	430.22	382.23
Wheat	100.00	200.00	300.00
Soybean Meal	380.98	283.92	232.77
Tallow/animal fat	22.96	23.86	25.23
L-Lysine Hcl	3.21	2.47	2.05
DL-Methionine	2.90	1.95	2.09
DCP	20.19	17.54	15.83
CaCo3	11.28	11.95	11.81
NaCl	2.73	2.59	2.49
Minerals premix*	2.50	2.50	2.50
Vitamin premix**	2.50	2.50	2.50
L-Threonine	2.80	2.80	2.80
Salt	2.00	2.00	2.00
Limestone	11.30	11.30	11.30
Sodium bicarbonate	4.40	4.40	4.40
Total	1000	1000	1000
Analyzed composition			
ME, kcal/kg	3025	3150	3200
Crude protein,%	22	21	19
Calcium,%	1.05	0.9	0.85
P available,%	0.5	0.45	0.42
Methionine,%	0.51	0.45	0.41
Lysine,%	1.43	1.24	1.09
Methionine + Cystine,%	1.07	0.95	0.86
Threonine	0.94	0.83	0.74

*Mineral premix provided per kilogram of diet, manganese, 55 mg;zinc, 50 mg; iron, 80 mg; copper, 5 mg; selenium, 0.1 mg; iodine, 0.36mg; sodium, 1.6 g.

**Vitamin premix provided per kilogram of diet, retinylacetate, 8,250 IU; cholecalcipherol 1,000 IU; dl-α-tocopherol, 11 IU; cyanocobalamin,0.012 mg; phylloquinone, 1.1 mg; niacin, 53 mg; choline, 1,020 mg; folacin, 0.75 mg; biotin, 0.25 mg; riboflavin, 5.5 mg.

To investigate the immune system of broiler chickens, the HI and SRBC tests were used. The separation of erythrocytes and injection of SRBC were conducted based on the presented method by Haghighi *et al.,* (2010). From each replicate, a bird was selected and 1 ml per kg of live weight of chickens of sheep red blood cells (SRBC) was injected into the pectoral muscle at the age of 28 years old. 7 days after the injection, blood sampling from the brachial vein of the same birds as 2 ml was conducted and blood serum was separated by centrifugation at 3000g (Universal

centrifuge type L BH-1200/ Netherlands) for 10 min. Amount of antibody titers against SRBC in serum obtained using the Haemagglutination Activity test (HA) and concentration of Newcastle disease vaccine titers obtained by Haemagglutination Inhibition test (HI) was determined. Animal handling and experimental procedures were performed according to the Guide for the Care and Use of laboratory animals by the National Institutes of Health (USA) and the current laws of the Iranian government for animal care.

Quantitative Real-Time PCR Analysis

To investigate the gene expression of Interleukin II, a bird was selected from each replicate and at the end of the period, as 1-2 ml of blood samples were taken from the venous vessel under the wing. Samples were transferred to the laboratory immediately after being coded and were kept there at the temperature -20 C° until DNA extraction. DNA extraction was conducted using the modified salting-out method developed by Miller *et al.*, (1998). To extract RNA, AccuzolTM (Cat. No. K-2102, Korea BIONEER) kit was used.

To normalize data and also delete the effect of possible errors, the housekeeping gene (Beta-actin) was used. To design IL-2 gene-specific primers, firstly the full sequence and length of the cDNA fragment were extracted from the NCBI website. Then, using the Online Primer 3 software program, for amplification of fragments with a length of 135 bp, required premiers were designed (Table 3).

Table 3: Primer sequences (5→3) used in real-time PCR

Primer		Sequence	Products (bps)
IL2	Forward	TGCAGTGTTACCTGGGAGAA	135
	Reverse	CTTGCATTCACTTCCGGTGT	
ß-Actin	Forward	CCGCTCTATGAAGGCTACGC	128
	Reverse	CTCTCGGCTGTGGTGGTGAA	

For making cDNA, QuantiNova^{TM} SYBRGreen PCR, QIAGEN kit was used. To draw the standard curve, six different dilutions of cDNA were prepared. For each target sample gene, a sample of Beta-actin was considered. Then, the target gene, beta-actin, and standards were put in the device Corbett Rotor-Gene 3000. Thermal cycling has stages of primary activation (95 °C for five min.), string isolation (95 °C for 15 sec.), and amplification (60 °C for 40 sec.).

To ensure the existence of the desired amplified fragments made in PCR, in the end, amplified products were loaded on agarose gel 2% (figure 1).

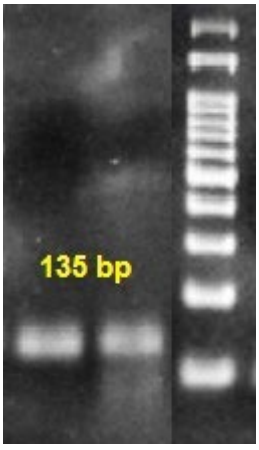

Figure 1: The expression qRT- PCR product on agarose gel 2% and the 135 bp fragment of the Interleukin-2

To determine the Real-Time quantity, colors such as SYBRGreen were used. Levels of the IL-2 gene are calculated with Livak's method (Livak and Schmittgen, 2001). In this method, firstly, the mean CT of target genes and beta-actin genes was calculated. Then, the value of ΔC_T ($\Delta C_{T\ (test)}$) was obtained from the difference of CT of target genes from the CT of the beta-actin genes. The first treatment (control 1, without injection) was considered as calibrator and the value of the ΔC_T related to this treatment was subtracted from the value of the ΔCT of other treatments. The obtained value is the $\Delta\Delta C_T$. then, the relative gene expression of IL-2 with the formula $2^{-\Delta\Delta CT}$ was calculated in the Excel software program. The software was used in the device Real-Time PCR (Rotor-Gene 6000 Series Software 1.7) for primary analysis of the data of the gene expression.

Statistical analysis

The experimental data were analyzed using MSTATC software in a completely randomized design ($Yij = \mu + Ti + eij$) and means were compared using Duncan's multiple range test at the significance level 5%.

RESULTS AND DISCUSSION

Immune

The results related to the effect of Thymolina on the immune system are represented in table 4. The highest degree of antibody in the SRBC test was related to the 0.5% Thymolina treatment and the least is related to the 2% Thymolina treatment. The highest degree of Newcastle titers in the HI test was related to the 2% Thymolina treatment and the lowest one was related to the 2% Thymolina treatment and the lowest one was related to the 1% Thymolina treatment. The results indicate that Newcastle titers and SRBC have not experimented and no significant difference was observed among them ($P>0.05$). However, the degree of immune titers increased in Thymolina treatments.

Table 4: Effects of Thymolyna on immunity

Parameter	treatment				SEM	P. Value
	T1 (0)	T2 (0.5%)	T3 (1%)	T4 (2%)		
SRBC	1.33	1.66	1.66	0.66	0.052	0.2942
HI	4.667	4.333	5.333	5.667	1.054	0.936

Experiment treatments cause an increase in the degree of Newcastle and SRBC Antibody Titers and result in improving the immune system. The increase in the degree of Newcastle Antibody Titers results from physiological improvement and the effect of thymol and carvacrol available in essences are factors of the immune system. The results obtained from this experiment is consistent with the results of Tollba *et al.*, 2011; Sadeghi *et al.*, 2011; Dong *et al.*, 2007, but inconsistent with those of Al-Ankari *et al.*, 2004; Jafari *et al.*, 2008; Dorhoi *et al.*, 2006; Toghyani *et al.*, 2010.

The lack of observing the effect on the immune system can be attributed to a kind of effective ingredient available in essences or phytogenic compounds, the doses used, or breeding conditions.

The increase in SRBC Antibody Titers resulting from the active compounds of medicinal plants such as polysaccharides, glycoproteins, and caffeic acid derivatives and alcids can modify and improve the immune system and causes the stimulation of phagocytic activity; therefore, it can stimulate the host immune response (Gudev *et al.*, 2004). In addition, herbal essences stimulate the immune response in bodies via the increase in the activity of vitamin C (Cook and Samman, 1996).

Since the desired compound is full of thymol and Phenolic compounds, it can be concluded that it causes stimulation and regulation of the immune system. Undoubtedly, with time, due to the elimination of the antigen and its effects in the body as well as the short half-life of the antibody against the antigen, the SRBC will be weaker at the end of the period. The results obtained from this experiment are consistent with the results of Mathivanan, & Kalaiarasi, 2007; Ghalamkari *et al*, 2011; Yakhkeshi *et al.*, 2011.

Stimulation or reinforcement of the immune system refers to the activation of parts of the immune system in the body by some external factors. These issues result in the creation of better immune against infectious microorganisms and toxins. These factors reinforce the immune mechanisms of the body and can reinforce passive immune responses in broiler chickens. After vaccination, achieving the maximum level of immune depends on a lot of factors such as birds' health. Not using Thymolina in the control treatment results in the reduction of Newcastle viruses and the observation of the minimum antibody titers against the primary and secondary immune responses.

Gene expression of interleukin-2

The results obtained from the gene expression of interleukin-2 are illustrated in table 5. The highest degree of the gene expression of interleukin-2 was related to the 2% Thymolina treatment and the lowest one was related to the control treatment. The results indicate that experiment treatments are influenced and significant difference was observed among treatments (P<0.05).

Table 5. Effects of Thymolyna on Gene expression IL2

Parameter	treatment				SEM	P. Value
	T1 (0)	T2 (0.5%)	T3 (1%)	T4 (2%)		
IL2	1.049 ^{c}	3.938 ^{b}	0.023 ^{c}	8.499 ^{a}	2.458	<0.0001

^{a,b,c}Means values within a row with different superscripts different significantly ($P < 0.05$).

The gene expression of interleukin-2 was significantly affected and there were significant differences among treatments (P<0.05). The most degree of gene expression of interleukin-2 was related to the2% Thymolina treatment and the lowest one was related to the 1% Thymolina treatment. Interleukin is a lymphokine that is the control some of the aspects of immune responses with the signal transmission between white blood cells and lymphocytes. Interleukins are cytokines that regulate relations between lymphocytes and other leukocytes. Interleukin II, by advancing the cell cycle towards the synthesis of cyclins via the analysis of P27, prevents the cell cycle process. Interleukin II causes the increase in the production of executive cytokines gamma – IFN and interleukin IV from T cells. Interleukin II causes the amplification and distinction of NK cells and increases the activity of cytotoxicity activity. Interleukin II is a factor of growth and stimulation of antibody synthesis for B cells. In the present research, with the increase of the degree of using Thymolina, gene expression of interleukin-2 increases and this issue causes the increase in the immune system. The results obtained from this experiment is consistent with the results of Shao *et al.*, 2007; Huang and Liu (2008); Xu *et al.*, 1997 and Zhang *et al.*, 2005.

CONCLUSION

Results indicated that using Thymolina in the diet of broiler chickens causes improvements in the immune system by increasing the gene expression of IL-2. Therefore, it can be effective on the immune responses of broiler chickens and cause the improvement and effective immune system in broiler chickens. In general, the results of experiments of the present study indicated the use of Thymolina as herbal additives which can be replaced by the growth-promoting antibiotics without side effects.

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