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Effect of Feeding Oak Acorn on Expression of *IL-2*, *IL-13* and *IFN-γ* Genes in Bursa Fabricius Tissue of Broiler Chickens (Short Communication)

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Abstract

Today, the use of oak as a replacement feed in poultry diets is well documented. However, oak contains polyphenol compounds (tannins) as anti-nutrient factors resulted in limited usage in the poultry rations. Generally, consumption of feeds containing tannin could affect gene expression level of immune system. Therefore, the aim of this study was to investigate the effect of different levels of oak acorn on the expression of interferon-γ (*IFN-γ*), interleukin-2 (*IL-2*) and interleukin-13 (*IL-13*) genes in the bursa Fabricius tissue of broiler chickens. For this purpose, broiler chickens were fed with diets containing 0 (control), 15% and 20% oak acorn in 6 weeks experimental period. Total RNA was extracted from bursa Fabricius tissue of 6 chickens for each treatment (18 broiler chickens) on day 42. To investigate the expression of *IL-2, IL-13 and IFN-γ* genes, their expressions were normalized with β-actin gene as a reference gene. REST, 2009, V2.0.13 software was used for analysis of gene expression data. The results showed that the expression levels of *IL-2* and *IL-13* genes in bursa Fabricius tissue were not significantly different for 15% oak acorn treatment compare to control group, but the mRNA expression levels of *IL-2* and *IL-13* were significantly lower in 20% of oak acorn in respect to control and 15% oak acorn diets ($P < 0.05$). However, expression of *IFN-γ* gene was not observed in any samples of the three treatments. According to the results of this study, it seems that increasing the amount of oak acorn in diet of broiler chicken with increase tannin content most likely could suppress the expression of IL-2 and IL-13 genes in bursa Fabricius tissue.

Introduction

Oak tree is one of native plants of the Zagros region in Iran producing a lot of fruits. Carbohydrates, starch in particular, make up most of the oak acorn and has the potential for using as a source of energy in the poultry diet. Oak acorn contains nutritional components and also various phenolic compounds such as tannins, gallic acid, ellagic acid, and different derivatives of galloyl and hexa-hydroxy-diphenoyl (Rakic *et al*., 2007). Oak acorn is one of the richest plants in terms of the amount of phenolic compounds and tannin and due to flavonoids compounds it has anti-inflammatory properties. Phenolic compounds can modulate the activity and transcription of enzymes (Kumari and Jain, 2012).

Flavonoids also show different biochemical and pharmacological functions that influence on activity of immune and inflammatory cells (Garcia-Lafuente *et al*., 2009). One of the most important properties of phenolic compounds is their ability to bind to positively charged groups in the structure of proteins and amino acids and thus reduce the bioavailability of these compounds (Deshpande, 2002). It has been reported that chickens fed with a high percentage of tannic acid reduces the rate of growth and the relative weight of bursa Fabricius, thymus and spleen indicating that polyphenol compounds may disrupt the normal growth (Marzo *et al*., 1990). Phenolic

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compounds contain numerous hydroxyl groups in their structure, a characteristic that enable them to stimulate or suppress the immune system. The hydroxyl groups can influence enzyme or electron-transfer systems, lead to immunomodulation of specific responses, in particular phagocytosis (Park *et al*., 2011). The immune system cells secrete a very various number of cytokines that are involved in the regulation of immune responses and important cellular functions such as survival, proliferation and differentiation (Canon, 2000; Oppenheim, 2001). The different expression of the cytokines in the body indicates the type of Th1 or Th2 immune response which itself has an important role in pathogenesis. The most important cytokines of Th1 cells are IFN-γ and IL-2, while IL-13 is a Th2 cytokine component (Libetta *et al*., 2011).

Nowadays, it is clear that there is apparent relationship between components of diet and functions of immune system (Ramiro *et al*., 2005). In the current researches on this topic, the major focus is to modulate the immune system of healthy people by nutrition to improve general health. For example, there are some reports that adding certain nutrients, especially those have antioxidant properties, may lead to improve immune function (Kelly and Bendich, 1996; Meydani *et al*., 1998). Considering the antioxidant effects of tannins, it can be expected that oak acorn can affect the immune system of broiler chickens. Therefore, the aim of this study was to investigate the effects of different levels of oak acorn on expression of *IL-2, IL-13* and *IFN-γ* genes in bursa Fabricius tissue of broiler chickens.

Materials and methods

In this study, 264 one-day-old chickens (male and female of Cobb 500) were used in a completely randomized design with three treatments and four repeats. The first treatment was fed with diet based on maize-soybean meal (without oak acorn) and considered as control diet, the second and third treatments contained 15% and 20% oak acorn, respectively. Diets were formulated according to the recommended nutritional values for broilers by NRC (1994) for the starting (days 1 to 21) and finishing periods (days 22 to 42) and using UFFDA software.

At the age 42 days, eighteen broiler chicken were selected randomly (6 chicks from each treatment) and their bursa Fabricius tissue were harvested. For extraction of RNA, small pieces of bursa Fabricius tissue were removed from each chicken and immediately stored in the nitrogen tank until the start of the laboratory experiment. Extraction of total RNA was carried out from 100 mg bursa Fabricius tissue using RNX-Plus solution (CinnaGen) according to its instructions. The quality and quantity of extracted RNA were evaluated by agarose gel electrophoresis and spectrophotometer. First single strand DNA was synthesized using AccuPower® CycleScript RT Premix (Bioneer) and then the quality of the obtained cDNA was evaluated using 1% agarose gel.

Specific primers were used to investigate the expression of target and reference genes (Table 1). In the quantitative determination method of the gene expression, correction of experimental changes is necessary. For this purpose, an internal control gene, i.e. β-Actin, was used. Real-time PCR was performed using CFX96 (BIORAD, USA) and *HotTaq EvaGreen qPCR* kit (Cinnagen) according to the manufacturer's instructions and reactions were performed in duplicate for all samples.

The amplification program used in the real time PCR reaction for target genes was 50° C for 2 minutes, 95 ° C for 10 minutes, 95 ° C for 15 seconds and 60 ° C for 1 minute, and the last two steps were repeated 40 times. This program for β-Actin gene was 5 minutes at 95 °C, 30 seconds at 94 °C, 30 seconds at 56 °C, and 30 seconds at 72 ° C, and the two to four steps were repeated in 45 cycles. Finally, comparison of gene expression at age of days 42 with 15% and 20% oak acorn treatment was performed using REST, 2009, V2.0.13 software and based on Ct values obtained from real time PCR with respect to the control group (Pfaffl *et al*., 2002).

Table1. The sequences, access numbers and locations of primers

Primer	Sequence	Size bp	Gene Bank ID	Reference
INF-γ	F: 5′- AAGTCAAAGCCGCACATCAAAC-3′ R: 5′- CTGGATTCTCAAGTCGTTCATCG-3′	132	X99774.1	Haiwen *et al.* (2010)
IL-2	F: 5′-TTCTGGGACCACTGTATGCTCTT-3′ R: 5′-TACCGACAAAGTGAGAATCAATCAG-3′	129	AF000631.1	Haiwen *et al.* (2010)
IL-13	F: 5′- CTGCCCTTGCTCTCCTCTGT-3′ R: 5′- CCTGCACTCCTCTGTTGAGCTT-3′	123	AJ621250.1	Haiwen *et al.* (2010)
β-actin	F: 5′- CTGTGCCCATCTATGAAGGCTA-3′ R: 5′-ATTTCTCTCTCGGCTGTGGTG-3′	139	NM_205518	Yang *et al.* (2013)

Results

Figure 1 shows amplified fragments for *IL-2* and *IL-13* and β-actin genes. As shown, the size of the amplified fragments for *IL-2*, *IL-13* and β-actin genes were about 129, 123 and 139 bp, respectively; indicating that, the accuracy of amplification for desired fragments was confirmed. However, interferon gamma gene was not amplified for all samples.

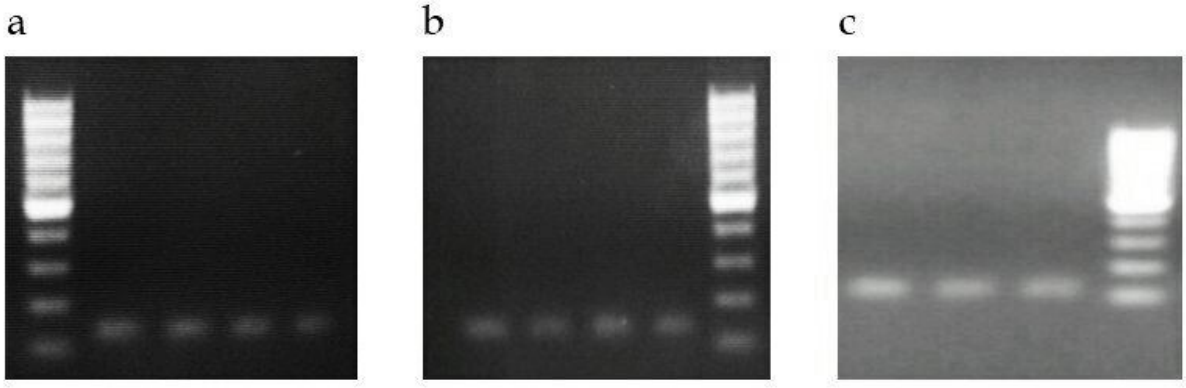

Figure 1. Amplified fragments using primers along with 100 bp ladder. a) amplified fragment of IL-13 gene; b) amplified fragment of IL-2 gene; c) amplified fragment of β-actin gene.

The mRNA expression of *IL-2* and *IL-13* genes for 15% and 20% oak acorn treatments on day 42 are presented as folds induction relative to the control in Table 2. As it shown, the expression of *IL-2* gene in 15% oak acorn treatment was not significantly different in compared to control treatment ($P = 0.54$), but the expression of this gene in the treatment of 20% oak acorn showed a significant reduction relative to oak acorn free treatment (control group) in a way that its expression was about 0.12 times lower than the control group ($P = 0.04$).

Table 2. Gene expression levels of IL-2 and IL-13 in broiler chickens fed with 15 and 20% oak acorn

Gene	Treatment	Expression rates	P value	Results
Interleukine-2	15% oak acorn	0.70	0.54	-
	20% oak acorn	0.13*	0.04	Down
Interleukine-13	15% oak acorn	0.85	0.79	-
	20% oak acorn	0.10*	0.03	Down

In addition, no significant difference was found between expression of *IL-13* gene in 15% oak acorn compare to control treatment ($P = 0.79$), while the mRNA expression of interleukin-13 was significantly lower in the 20% oak acorn compare to control treatment ($P = 0.03$) and its expression was 0.10 times lower than control group (Figure 2).

The expression results of *IL-2* and *IL-13* genes in the 20% oak acorn relative to 15% treatment were significantly lower (Table 3). The expression levels of *IL-2* and *IL-13* in 20% treatment were 0.18 and 0.12 folds lower than 15% oak acorn treatment, respectively.

Table 3. Gene expression levels of IL-2 and IL-13 in broiler chicken fed with 20% oak acorn compare to 15% oak acorn

Gene	Treatment	Expression rates	P value	Results
Interleukine-2	20% oak acorn	0.18*	0.04	Down
Interleukine-13	20% oak acorn	0.12*	0.01	Down

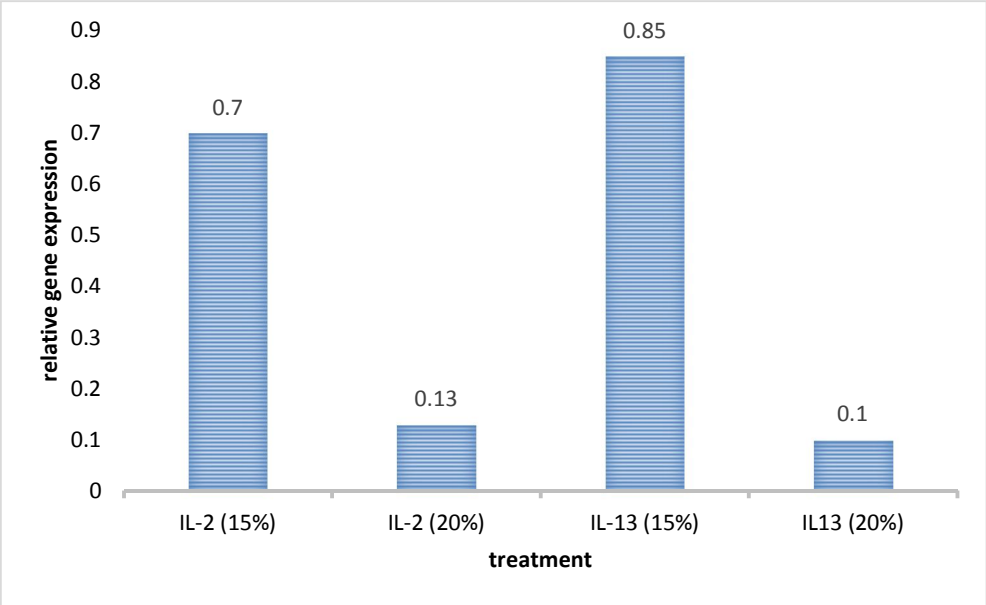

Figure 2. Expression of *IL-2* and *IL-13* in broiler chickens fed diets containing oak acorn compare to control group on day 42.

Discussion

In the present study, the gene expression levels of *IL-2* and *IL-13* on day 42 for 15% oak acorn treatment were not different from control group, but with increasing the amount of oak acorn in 20% treatment, expression levels of both genes were significantly lower than control and 15% oak acorn treatments. Regarding the effect of tannic acid on immune system of broiler chickens, it has been observed that the diet administration of tannic acid caused a significant reduction in weight of the bursa Fabricius, thymus and spleen in broiler chickens (Marzo *et al*. (1990). In addition, the total white blood cells, absolute lymphocytes and levels of M and G immunoglobulins were reduced in chickens fed with tannic acid (Marzo *et al*., 1990). They have suggested that tannic acid could impair development of immune system in growing chickens, which its severity depends on the amount of added tannic acid (Marzo *et al*., 1990). Reduction of the bursa Fabricius weight seems to be associated to genes expression of immune system, which is consistent with results of the present study. Generally, it is suggested that phenolic compounds, especially tannins, block the absorption of sugars and amino acids in the intestine (Santidrian and Mano, 1989), which reduces the availability of amino acids in diets containing tannin, and reduces protein synthesis in lymphoid tissues (Marzo *et al*., 1990). Since antibodies and interleukins are the most important serum proteins associated with the immune system, it is expected that reduction of serum proteins could lead to suppression of the immune system. Diet containing 20% oak acorn due to having more phenolic compounds and anti-nutrients substances, such as tannins, is not palatable and leads to reduced feed intake. On the other hand, synthesis of interleukins requires protein substances; it seems that reducing feed intake can reduce interleukins production, which is consistent with the results obtained in this study. In this regard, Hamou *et al*. (2012) reported that the presence of tannin in oak acorn decreases feed intake in broiler chicken.

In fact, it is confirmed that phenolic compounds inhibit expression and secretion of cytokines. In present study, the expression of Th1 (IL-2) and Th2 (IL-13) cytokines significantly decreased in broiler chicken fed diets contain oak acorn. In addition, reduction of IL-2 secretion was observed in the lymphocyte cells treated with cocoa extract that results in a cell to be less sensitive to the autocrine effect of IL-2 (Ramiro *et al*. 2005). The secretion of IL-2 was prevented in human peripheral blood cells exposed to cocoa liquor polyphenol (Sanbongi *et al*., 1997). Furthermore, extraction of crude cocoa reduced transcription of *IL-2* gene in human peripheral blood lymphocytes which is consistent with the results of the present study in broiler chickens. Tomita *et al*. (2002) reported that tea polyphenol inhibits the production of Th1 and Th2 cytokines in $CD4^+$ T cells. They found that *IL-2* gene expression, IL-2 secretion, and the activation of NF-kappaB were suppressed in $CD4^+$ T cells of murine spleens by tea pigments. They also observed that tea pigments suppressed the expression of *IL-4* and *IL-5* genes, cytokines produced by Th2 cells. Since IL-13 is structurally and biologically similar to IL-4, it seems that IL-13 should be decreased as well. Secretion levels of IL-6 and TNF-α reduced in LPS-stimulated RAW 264.7 macrophages treated by flavonoids (Mueller *et al*., 2010). Production of IL-1β, TNF-α, IL-6, and IL-12 significantly decreased in human periodontal ligament cells incubated with

apigenin flavonoid (Jeong *et al*., 2009). In addition, Tunon *et al.* (2009) reported that IL-8 production was suppressed in human nasal fibroblasts and A549 epithelial cells exposed to green tea polyphenol. Transcription and secretion of TNF-α, IL-1 or IL-6 have been shown to be suppressed in RBL-2H3 cells by flavonoids (Park *et al*., 2008). Tea-derived catechins decrease the production of IL-10 in human leukocytes (Crouvezier *et al*., 2001). Also, i*n vitro* studies have shown that epigallocatechin-3-gallate (EGCG), the major active component of green tea, inhibits proliferation of T cell derived from spleen cellsof mice. They also found that dose dependent of EGCG inhibited division of T cell and progression of cell cycle. In addition, EGCG supplementation led tolower expression of IL-2 receptor and higher accumulation of IL-2 (Wu *et al*., 2009).

Generally, transcription factors, such as NF-kB and AP-1, have important roles in regulation of genes involved in immune responses such as *IL-2* and *IFN-γ* (Fujioka *et al*., 2004; Park *et al*., 2000; Mao *et al*., 2000). On the other hand, flavonoids compounds can regulate activity of transcriptional factors, like NF-kB and Nrf-2, in inflammatory and antioxidant pathways (Maroon *et al*., 2010). Therefore, it is suggested that transcription factors like NF-kB may alter the profile of cytokine expression in cultured cells exposed to phenolic compounds (Han *et al*., 1998; Zhao *et al*., 1999). For example, T cells stimulation led to activation of transcription factors NF-kB, NF-AT and NF (P). The transcription factor NF-AT has been involved in the expression regulation of IL-2, IFN-γ and IL-5 genes (Campbell *et al*., 1996).

In the present study, the interferon-γ gene was not expressed in any the treatment groups. Interferon-γ is produced by stimulating mitogens and antigens. Therefore, normal cells until have not been induced for interferon-γ synthesis, do not express it and viral infections are one of the strong factors of interferon production. Considering the fact that in this study, broiler chickens were not affected by the immune challenge and oak acorn also is not containing mitogens compounds, it seems that oak acorn could not stimulate the expression of interferon-γ gene.

Conclusion

Based on results of this study, use of oak acorn in diet of broiler chickens would affect the expression of immune system genes and its effect depends on dietary level of oak acorn. In this way, expression of interleukin-2 and interleukin-13 genes were suppressed in bursa Fabricius tissue of broiler in treatment of 20% oak acorn, while consumption of lower oak acorn level (15%) did not influence on the expression of the two studied genes.

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