

Short Communication

Effect of thyme (*Zataria multiflora*) extract and probiotic (Broilact) feeding on IGF-I, IGF-II and IGF- I R gene expression of liver in broiler chickens

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ABSTRACT:

This study aimed to investigate the effect of *Z. multiflora* extract and probiotic (Broilact) feeding on IGF-I, IGF-II and IGF- I R gene expression of liver in broiler chickens. Therefore, Insulin like Growth Factor- 1 (IGF- I), Insulin like Growth Factor- 2 (IGF- II) and IGF- I receptors gene expression were measured and found improved. One hundred and eight one-day old Ross male broiler by were randomly allocated in three groups viz: control group (CTRL), group supplemented with *Zataria multiflora* extract (Thy) and probiotic feed (Pro) group. Each group had three replicates of 12 broilers. The birds received a corn- soybean based diet. IGF- I, IGF- II and IGF- I R gene expression were determined in liver. The results of this study showed that the amount of thyme extract used in this experiment has no effect on thyroid hormones concentrations. At the 42nd day of age IGF- I and IGF- II gene expressions in liver have not significantly changed in treatments ($P>0.05$). IGF- I R gene expression in Pro birds significantly increased when compared with CTRL and Thy birds ($P<0.05$).

Keywords:

IGF- I gene expression, Probiotic, Thyme extract, Broiler.

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Article Citation:

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Journal of Research in Ecology (2017) 5(2): 830-837

Dates:

Received: 13 May 2017 Accepted: 14 July 2017 Published: 22 July 2017

Web Address:

[http://ecologyresearch.info/
documents/EC0386.pdf](http://ecologyresearch.info/documents/EC0386.pdf)

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INTRODUCTION

During the recent years, broiler farming has seen a remarkable growth regarding its role and importance in providing the animal protein needed for human and has changed into a large industry. Using antibiotics has caused serious problems for public and animal health due to producing resistant bacterial species and possibility for transfer of this resistance to human and disturbing normal alimentary tract flora, and this issue has worried the consumers. Thus, the necessity for limiting consumption of lots of antibiotics and growth-improving agents, the phytogetic compounds and probiotics have widely been investigated and applied (Bai *et al.*, 2013; Bustin *et al.*, 2005; Mountzouris *et al.*, 2010; NRC, 1994). Herbal extracts may enhance appetite through stimulating olfaction and taste. In addition, they elevate absorption of nutrients by increasing digestive secretions, stimulating enzymatic activities, and facilitating transfer mechanisms. They also have an important role in inhibiting bacterial and fungal growth, and stabilizing the microbial flora of alimentary tract (Mountzouris *et al.*, 2009). Thyme could be named among the most important extracts used in animal and poultry food, which stimulates growth, reduces feed conversion coefficient, and imposes some other effects such as antioxidant impact because of some of its ingredients like thymol (Lee *et al.*, 2003; Livak and Schmittgen, 2001). Lots of information are available regarding *Thymus vulgaris*. Another notable species of this plant is *Zataria multiflora* from the Lamiaceae family (Aeschbach *et al.*, 1994).

Nowadays, probiotics are increasingly being used in the poultry industry because of improving the intestinal microbial flora, reducing diarrhea occurrence, increasing immunity level, the ability to compete pathogenic bacteria, inducing resistance against stress, and producing some enzymes. Adding many of these microorganisms leads to weight gain during the first week of farming in broiler chickens. Moreover, the probiotics

might control the pathogenic agents in poultry alimentary tract, hence they are effective in improving nutritional function, and also increasing feed intake and enhancing food conversion coefficient, and increasing the body weight as a result (Ragione *et al.*, 2004; Schneitz *et al.*, 1998). Nurmi and Rantala (1973) have tried to take an effective step for controlling Salmonella infections in broilers by presenting competitive exclusion hypothesis during the beginning years of 70s, but it was found out that using probiotics will result in the protection against bacterial infections and even growth elevation (Aho *et al.*, 1992; Elwinger *et al.*, 1992; Goren *et al.*, 1984; Hakkinen and Schneitz, 1996; Palmu and Camelin, 1997; Soerjadi-Liem *et al.*, 1984).

Broilact was one of the first commercial productions regarding this hypothesis, which was manufactured in Orion Company in Finland, and was produced as liquid in Finland and Sweden from 1987 until 1994; since then, it has been presented as solid powder. In fact, Broilact is a mixture of 32 bacteria chosen from cecal contents of healthy adult hen which is capable of binding to intestinal mucus (Palmu and Camelin, 1997; Schneitz and Hakkinen, 1998).

In addition to competitive exclusion properties of Broilact, it was determined that these microorganisms are problematic for the activity of pathogenic bacteria like Salmonella because of producing volatile fatty acids. Besides, Schneitz *et al.* (1998) demonstrated that

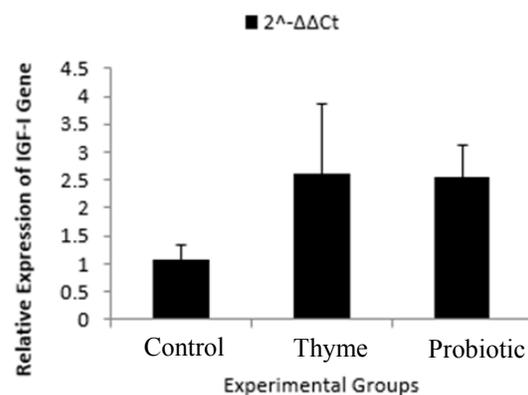


Figure 1. IGF-I gene expression at the end of 42 days under experimental factors using $2^{-\Delta\Delta Ct}$ method.

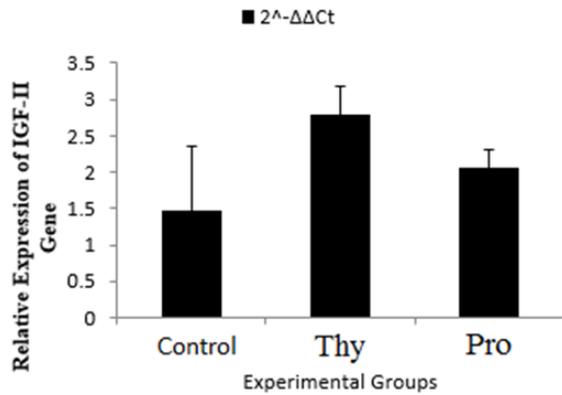


Figure 2. IGF-II gene expression at the end of 42 days under experimental factors using $2^{-\Delta\Delta Ct}$ method.

using this probiotic increases digestibility of organic materials, enhances retention of nitrogenous substances, and finally improves coefficient of conversion and growth, in addition to reducing chickens' mortality. Part of this improvement in efficiency has been attributed to the increase fermentation in intestinal contents. Furthermore, considering their competitive exclusion function, probiotics allow rapid homing of normal and complete flora at the beginning of chickens' lives, and inhibit adhesion of pathogenic bacteria to intestinal membrane by forming a protective cover (Nurmi and Rantala, 1973).

Insulin-like growth factors type I and II (IGF-I and IGF-II) are polypeptides and have structural and functional similarities to insulin hormone. However, these growth factors are different in various bird species compared to mammals, regarding both number and amino acids sequence. These hormones play an important role in animals' growth beside growth hormone, thyroid hormones, and insulin hormone (Kadlec *et al.*, 2011).

Researches indicated that lots of growth hormone's functions in birds are performed through insulin-like growth factors like mammals, some of which include increasing glucose and amino acids metabolism, DNA synthesis, different body cells proliferation, and also growth regulation (McMurtry, 1998).

Unlike fetal period that mRNA and synthesis stages of these growth factors have been observed in

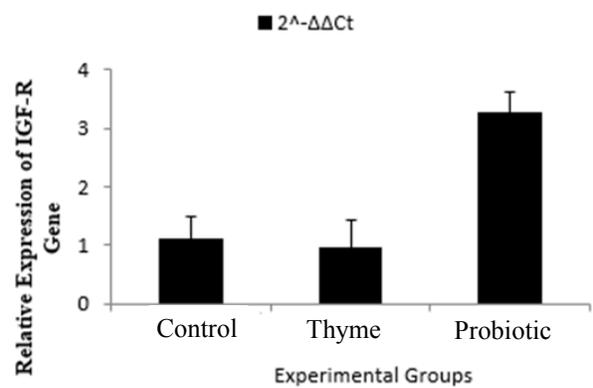


Figure 3. IGF-IR gene expression at the end of 42 days under experimental factors using $2^{-\Delta\Delta Ct}$ method.

brain, eye, and bone tissues of birds, hepatic cells and partly cardiac cells have the most role in producing these hormones after hatch (Kadlec *et al.*, 2011; Richards *et al.*, 2005).

Function of insulin-like growth factors is performed by their effects on their specific receptors (IGF-I Receptors) which are seen on the surface of most of the animal cells. According to the performed studies, there is no evidence of IGF-II receptors presence in chickens compared to mammals, and IGF-II plays its role by affecting IGF-I R just like IGF-I. These receptors belong to a large group of receptors known as tyrosine kinase, and insulin receptors are also classified in this group. Activation of these receptors lead into development and hypertrophy of some cells like skeletal muscles and other target tissues (McMurtry *et al.*, 1997).

No extensive researches have been conducted regarding evaluation of direct impacts of thyme extract and the mentioned probiotic on endocrine system, especially insulin-like growth factors and their receptors which have an important role in metabolism and growth of chickens, and since gene expression is the first step in synthesis of any substance such as hormones, we have tried to investigate the effects of Shirazi thyme extract and probiotic on expression of IGF-I, IGF-II, and IGF-I R genes in liver of male broiler chickens.

Table 1. Characteristics of the primers utilized in this research

Gene	Record No. in Gene Bank	Primer Sequence (5'-3')	Direction	Band Size (bp)
IGF-I	M32791	TggCCTgTgTTTgCTTACCTT	Forward	301
		TTCCTTTTgTgCTTTTggCAT	Reverse	
IGF-I R	S40818	CTCTCCCCAACCTCACggTCA	Forward	274
		gCTTCTCCTCCATCgTTCCTgg	Reverse	
IGF-II	NM_00103034 2.1	TgTggAggAgTgCTgCTTTC	Forward	101
		gggAggTggCggAgAggTCA	Reverse	
β -Actin	L08165	gAgAAATTgTgCgTgACATCA	Forward	152
		CCTgAACCTCTCATTgCCA	Reverse	

MATERIALS AND METHODS

This experiment was performed in the agriculture and industry complex of Amol. At the time of entering of the chickens to farming salon, the chickens were separated on the basis of gender difference; and the roosters were chosen for the experiment. Then the chickens were weighed one by one and the those chickens over 44.5 grams were chosen and randomly divided into the pans. Permanent lighting programs were used in this study. During the whole 42-days program, antibiotics were not used in any of the groups, and the mortality in each experimental unit was recorded daily (Kadlec *et al.*, 2011). This research was performed as a totally randomized design with three treatments and three repeats with twelve observations in each repeat, so there was nine experiment units with 12 broiler one-day rooster chickens of Ras breed (108 in total) were randomly put in the cages.

Experimental treatments included: Treatment 1 (control): basic ration, Treatment 2: basic ration + Shirazi thyme extract, Treatment 3: basic ration + probiotic. Nutritional requirements were regulated according to recommendations of NRC (1994). The rations were completely alike regarding the components and chemical composition and were just different in thyme extract/probiotic usage. On the age of 42 days, two birds were chosen from each experimental unit with weights close to the average of each pan, and were slaughtered immediately for obtaining liver tissue, and the samples of liver tissue from each repeat were immediately transferred to laboratory in -180°C , and were kept until gene

expression tests. All experimental procedures such as feeding, sampling based on professional ethics committee of Tehran Islamic Azad University, Science and Research, Iran.

The amount of thyme extract was chosen based on Lee *et al.* (2004), so that 100 ppm thymol existed in the ration. Regarding the result of Shirazi thyme extract analysis consumed in this research, and presence of 0.5% thymol in its composition, 20 grams of the extract was used for making concentration of 100 ppm from this phenolic compound in rations of chickens per each kilogram of consumed seed. The mentioned extract was mixed with the seed daily and was given to the chickens during all of the 42-days period, and the thyme extract containing seed was gathered from the troughs every day.

Preparation stages and consumption route for Broilact probiotic which is based on nonpathogenic bacteria including *Bacteroides*, *Porphyromonas*, *Eubacterium*, *Lactobacillus*, *Enterococcus* and *Escherichia*, have all been performed according to the guideline of its manufacturer (Orion) and the researches done by Palmu and Camelin (1997). Dry powder has been used in this study. In order to provide the amount of bacteria recommended by the manufacturer for the needed influence, one 2-grams packet of lyophilized dry powder probiotic with at least 10^{10} CFU in each gram was solved in 5 liters of water per each 2000 chickens, and the solution was given to the chickens during the first 4 hours of farming period so that each bird received 1 milligram of probiotic.

All the stages of molecular studies were performed in molecular genetic laboratory of animal science faculty of Sari agricultural university. Firstly, AccuZol solution (AccuZol-Catno K 3090) of Bionir Company was used in order to extract RNA from liver tissue. Extraction stages including homogenization of samples, isolation, RNA sedimentation, RNA washing, and RNA drying were all done based on the kit's guide. Then, the extracted RNA was kept at -70°C until cDNA synthesis, and after that the Quantifast Reverse Transcriptase kit of Kiagen company (Cat. No. 205311) was used to produce cDNA. The obtained cDNA was then kept at -70°C after solving it in ion-free sterile water until the next steps of the experiment (Figure 1).

In order to perform real time PCR, the Quantifast SYBER Green PCR kit of Thermo Scientific company (Lot. No. 00145251) was utilized. Using the Vector NTI software, specific primers were designed and then were made by Metabion Company. The characteristics of the used primers have been demonstrated in Table 1. The primers were used in concentration of 10 pmol/μL as mentioned in the protocol of QuantiFast SYBER Green PCR kit for real time PCR reaction.

In this study, ribosomal RNA (rRNA β-actin) was utilized as the reference gene (Bustin *et al.*, 2005; Livak and Schmittgen, 2001; Pfaffi, 2001). In order to measure the relative expression of IGF-I, IGF-II, and IGF-I R genes, real time PCR Rotor-Gene system of Corbett Company was used according to Livak and Schmittgen (2001) protocol.

Data of this study were analyzed as a completely randomized design using SAS software (SAS Institute Inc., 2003) and Duncan's test was used for comparing the means. The statistical model used is as follows:

$$Y_{ij} = \mu + \text{Treat}_i + e_{ij}$$

RESULTS AND DISCUSSION

The results of gene expression tests under experimental factors used in this research in the three experi-

mental groups including:

- Control group
- Shirazi thyme extract consuming group
- Probiotic consuming group

Considering the results of some researches which demonstrate that using thyme has led into improved conversion coefficient and weight gain in broiler chickens (Abdulkarimi *et al.*, 2011; Aliakbarpour *et al.*, 2012; Lee *et al.*, 2004) and also presence of various studies that are indicative of probiotics usage and impact on poultry growth enhancement (Ragione *et al.*, 2004; Schneitz and Hakkinen, 1998), this research was performed with the aim of investigating the influence of Shirazi thyme extract and probiotic on IGF-I, IGF-II, and IGF-I R genes expression in the livers of broiler rooster chickens.

Results of this evaluation showed that consuming Shirazi thyme extract in the amount used in this research does not affect expression of IGF-I, IGF-II, and IGF-I R genes in broiler rooster chickens' liver ($p > 0.05$) (Figure 2 and 3). Researches indicated that using thyme extract in broiler chickens has antioxidant and anti-bloat effects and improves the function of immune system, and also has positive effects on blood biochemical metabolites (Aho *et al.*, 1992). On the other hand, studies conducted by Motlagh *et al.* (2015) indicated that enhanced growth function due to consuming Shirazi thyme extract in broiler chickens has not been related to increase in hormones involved in growth such as thyroid hormones and growth hormone, and it is possible that thymol and other effective components in this extract improve growth through affecting appetite centers located in hypothalamus and other mechanisms effective on food receive.

According to the results obtained from this study, consuming the amount of probiotic used in this research has not affected the relative expression of insulin-like growth factors in the age of 42 days, but in-

creased expression of IGF-I R gene significantly compared to the group consuming thyme extract and also the control group. Studies demonstrated that growth of hormone causes growth in bones, muscles, and other body tissues both directly and indirectly by stimulating the production and secretion of somatomedins like IGF-I and IGF-II from tissues especially liver tissue (Huybrechts *et al.*, 1985).

Expression of the genes involved in the growth is in the fact the first stage of synthesis for these hormones, and also increase in receptors of these growth factors is the first step of getting affected in tissues in response to these hormones. Thus, molecular studies in this stage of protein synthesis could provide useful information about these factors because biological traits such as the mechanism by which hormones involved in growth are secreted are in fact a response of related gene behaviour under the influence of environmental factors.

Results of the studies indicated that improved digestion and absorption of nutrients due to probiotic consumption in birds could be an effective factor on biological growth-related factors (Mountzouris *et al.*, 2010; Schneitz *et al.*, 1998). Furthermore, according to the results of studies, probiotics have been reported to have positive influence on level of those hormones which affect growth such as thyroxin, and also on expression of growth hormone gene in liver of broiler chickens (Motlagh *et al.*, 2015). It seems that the increasing effect of the used probiotic on IGF-I receptors gene expression has resulted from the positive impact of consuming the probiotic used in this research on elevating the digestion and absorption of nutrients in alimentary tract and increasing retention of nitrogenous substances (Schneitz *et al.*, 1998).

Although limited studies have been performed regarding the effect of nutritional factors and probiotics on growth function and expression of various genes in birds (Aliakbarpour *et al.*, 2012; Horn *et al.*, 2009;

Mountzouris *et al.*, 2010), the biological impact mechanism of probiotics has not yet been reported (Chichlowski *et al.*, 2007; Mountzouris *et al.*, 2010), and unfortunately there are no reports available which demonstrate the direct effect mechanism for probiotics' influence on expression of genes involved in growth of birds. Therefore, considering the presence of limited reports about biological mechanism of nutritional factors and probiotics impact on growth, studying thymol and probiotic effect on expression of insulin-like growth factors and their receptors genes requires more extensive researches.

CONCLUSION

Based on the achieved results, IGF- I R gene expression in Pro birds significantly increased compared with CTRL and Thy birds ($P < 0.05$). Evaluation showed that consuming Shirazi thyme extract in the amount used in this research does not affect expression of IGF-I, IGF-II, and IGF-I R genes in broiler rooster chickens' liver ($p > 0.05$).

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