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Research Article

EFFECT OF THYME *(ZATARIA MULTIFLORA)* EXTRACT AND PROBIOTIC (BROILACT) FEEDING ON BLOOD THYROID HORMONES CONCENTRATION AND GROWTH HORMONE GENE EXPRESSION OF LIVER IN BROILER CHICKENS

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ABSTRACT
This study was conducted to examine the effects of thyme extract and probiotic on biological growth promoters. Therefore blood thyroid hormones (T3 and T4) concentration and growth hormone (GH) gene expression were measured. 108 one-day-old Ross male broiler were randomly allocated in 3 experimental treatments including: control- unsupplemented (CTRL), birds supplemented with Zataria multiflora extract (Thy) and probiotic in feed (Pro). Each treatment had 3 replicates of 12 broilers. The birds were fed on a corn- soybean based diet. T3 & T4 concentrations and GH gene expression in liver were determined. At 42 day of age T3 concentration not significantly changed in treatments ($P>0.05$). T4 concentration was significantly higher in Pro compared to Thy ($P<0.05$). GH gene expression in Pro birds significantly increased compared with CTRL and Thy birds ($P<0.05$) and thyme extract had no effect on GH gene expression and T3 and T4 ($P>0.05$). It may be concluded that dietary supplementation of probiotic may influence the biological growth promoters so T3 & T4 concentrations and GH gene expression in broiler and thyme extract had no effect on this biological parameters. Researchers indicated that some phytogenic extracts or probiotics improve growth performance.

Keywords: *Broiler, Thyroid Hormones, GH Gene Expression*

INTRODUCTION
Researchers in Previous studies demonstrated the positive effects of growth hormone and thyroid hormones on growth performance in broiler chickens (Lazar, 1993; McNabb and King, 1993; Huybrechts *et al.*, 1985). Today, some feed additives such as phytogenic extracts and probiotics are used as growth promoters in poultry nutrition (Abdulkarimi *et al.*, 2011; Al-Kassie *et al.*, 2009; Lee *et al.*, 2004; Peric *et al.*, 2010).

Probiotics are a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance (Fuller, 1989). Application of probiotic and phytogenic feed additives are alternative for antibiotic growth promoters (Bai *et al.*, 2013; Amad *et al.*, 2011). Probiotic bacteria can maintain the intestinal beneficial microorganisms and competitive exclusion against pathogenic bacteria (La Ragion *et al.*, 2004; Mountzouris *et al.*, 2012). Based on some previous studies probiotic supplementation reduced the colonization Clostridium. jejuni and Salmonella, improved intestinal immunity, feed efficiency, growth performance and meat quality (Bai *et al.*, 2013; Aliakbarpour *et al.*, 2012; Bansal *et al.*, 2011; Zhou *et al.*, 2010; Higgins *et al.*, 2008). According to previous investigations, phytogenic feed additives may improve intestinal communities, broiler performance; feed conversion ratio and poultry health (Kucukyilmaz *et al.*, 2012; Amad *et al.*, 2011). Thymol is a phytogenic components, is derived from thyme and exhibit antimicrobial activity (Lee *et al.*, 2003). According to Luna *et al.*, (2010), Thymol supplementation improved meat quality. Mode of action of thyme can be explained by antimicrobial activity (Lee *et al.*, 2003) or an improvement of intestinal nutrient digestibility (Amad *et al.*, 2011), but Peric *et al.*, (2010) indicated that stimulatory effects of phytogenic additives on growth performance in broiler chickens are not connected with the gut morphology and cecal microbial concentrations.

The mechanisms of action of phytogenic extracts and probiotics on growth performance are not completely clear (Peric *et al.*, 2010; Chichlowski *et al.*, 2007; Mountzoris *et al.*, 2010). The aim of this study was to investigate the potential of thyme extract and probiotic on biological growth promoters such as blood T3 and T4 concentrations and GH gene expression in liver.

MATERIAL AND METHODS

Birds, Management and Housing

One hundred and eight of one-day-old Ross male broiler chicks used in a completely randomized design with three treatments and three replicates and 12 birds per each. Thyme extract and commercial probiotic (Broilact) were used as feed additive at this experiment. All broiler chicks were randomly assigned into one of three treatments, including control On the basis of similar body weight. The Control group was fed ad lib with a commercial diet. Thy group was fed with control diet and Thyme (Zataria multiflora) extract. PRO group was fed with control diet and Probiotic in drinking water (first 4 hours). All of corn-soybean based dietary treatments were formulated to meet the NRC (1994) for starter (1 to 21 days), grower (22 to 35 days) and finisher (36 to 42 days) periods.

The probiotic, as listed by the manufacturer (Orion Corporation, Finland) , included mixture of selected chicken intestinal bacteria. According to manufacture protocol, 1mg of probiotic per bird in a dose volume of 0.3 ml given once (orally by gavage), on first 4 hours, on the first day at this experiment.

According to manufacture (Barij essence pharmaceutical Co, Kashan, Iran) 20 gr Thyme extract containing 0.5% thymol compound dissolved in oil and then gently mixed with the diet to arrive 100 mg/kg of thymol in the total diets for (Hashempour *et al.*, 2013; Lee *et al.*, 2004).

The experimental chicks were reared on wood shavings litter and assigned to a clean floor pen (2×1 m), with one hanging tube feeder and three nipple drinkers. The healthy program, house temperature and lighting schedule throughout the experiment were provided according to Ross broiler management guidelines.

Blood Sampling and Hormone Assays

Two birds from each pen were randomly chosen at the end of experiment (42 day of old) and Blood samples were collected from a wing Vein and then were placed in specific tubes and centrifuged at 3000 rpm under constant temperature of 4°C for 10 min (Refrigerated Centrifuge, Hettich. D-78532. Germany) and then immediately were used for T3 and T4 hormones analysis. T3 and T4 concentrations were measured by ELISA (Rayto Life and Analytical Sciences Co., Ltd. China) and commercial ELISA kits (Lot No 89001 & Lot No 90005, Pishtazan teb Company, Tehran, Iran, and RaytoRT-2100C) as described by Dai *et al.*, (2011).

Growth Hormone mRNA Quantification and Sample Collection

At the end of the study, two birds from each pen randomly selected and slaughtered. The liver was removed for GH mRNA quantification and frozen in liquid nitrogen container, then transported to the Molecular Genetics and Animal Biotechnology laboratory (Department of Animal Sciences, Sari Agricultural Sciences and Natural Resources University, Sari, Iran) and stored at -80°C until used for Real-Time qPCR assay.

Total RNA was extracted using Accuzol reagent from the liver segment according to the manufacturer's protocol (Bioneer, Cat. No. K3090). cDNA was synthesized from Quantifast Revears-Transcriptase kit (QIAGEN, Cat. N0. 205311). subsequently, qPCR was carried out with a specific primer pairs (forward: 5 GAG AAA TTG TGC GTG ACA TCA -3′; reverse: 5′- GCC AGG ACT GGA TGA GAA CC -3′) using Quanti Fast SYBER Green PCR kit (Thermo Scientific, Lot No 00145251). Specific primer pairs were designed by Vector NTI software and then were made by Metabion Inc, Co. At this experiment, β–actin RNA gene (L08165; forward: 5′- GAG AAA TTG TGC GTG ACA TCA -3′, reverse: 5′- CCT GAA CCT CTC ATT GCC A -3′) was chosen as a reference gene (Lian *et al.*, 2010).

Amplification of the chicken liver Pooled total RNA aliquots was performed for 45 cycles, which consisted of an initial activation step (95°C, 5 min), denaturation cycle (95°C, 10 s) and combined annealing and extension (60°C, 30 s). In each PCR run, preparation of standard curve was carried out by

serial dilution of pooled cDNA from samples. Product of Real time PCR on agarose gel (2%) is shown in figure 1. The relative expression ratio of Growth hormone as a target gene was normalized to β–actin RNA gene using 2-ΔΔct method (Livak and Schmittgen, 2001).

Statistical Analysis

A completely randomized design with 3 treatments was used. Data were analyzed by using the General Linear Models procedures of SAS 9.1 (SAS Institute Inc. 2003). Differences between means were tested using Duncan's multiple range test. The statements of statistical significance were based on $P<0.05$.

RESULTS AND DISCUSSION

Results

T3 & T4 Concentrations

The effects of feed additives on serum T3 & T4 concentrations are shown in Figure 2 & 3. In this study serum T3 concentration did not differ significantly among the treatments ($P>0.05$).

Serum T4 concentrations numerically was higher in CTRL group than Thy group, but this difference was not statistically significant ($P>0.05$). Serum T4 concentrations was significantly increased in probiotic treated birds compared to the group that was fed control a diet containing thyme extract ($P<0.05$). No significant differences were found in serum T4 concentrations between control birds and probiotic supplemented ($P>0.05$).

GH Gene Expression

The effects of feed additives on GH gene expression are shown in Figure 4. In this study the expression of GH mRNA numerically was higher in CTRL group than Thy group, but this difference was not statistically significant ($P>0.05$). Expression of GH mRNA was significantly increased in probiotic treated birds compared to the CTRL and Thy groups.

Discussion

Hormones secreted by the bird's thyroid gland, like mammals is effective to control metabolism as well as growth of body tissues (Lazar, 1993). Previous studies indicated that normal level of thyroid hormones indirectly affected bird's growth, due to the stimulatory effect of growth factors such as insulin like growth factor (McNabb and King, 1993). Based on the results of different studies, usage of thyme in broiler's diet improved feed conversion and increased weight of broilers (Abdulkarimi *et al.*, 2011; Al-Kassie *et al.*, 2009; Lee *et al.*, 2004). Similarly, several investigations showed that growth performance of broilers improved using probiotics (Peric *et al.*, 2010 and Zhi-gang *et al.*, 2014).

The results of this study showed that the amount of thyme extract used in this experiment has no effect on thyroid hormones concentrations. Also, T3 hormone concentration was not influenced by usage of probiotic. Cold weather is considered as the most important factor in stimulating the production of thyroid hormones as documented by Blahová *et al.*, (2007) and Stojević *et al.*, (2000). More than 90% of thyroid hormones produced by the thyroid gland of chickens are T4 and Most of T3 hormones are conversion of T4 to T3 in the pituitary gland (McNabb, 1999). The amount of thyme extract and probiotic used in this study had no significant effect on blood T3. Previously researchers reported that T3 hormone is less influenced by appetite stimulant and growth (McNabb, 1999). However, usage of probiotic significantly increased blood T4 compared to the Thy group. It seemed that increased effect of probiotic used in this study on production and secretion of T4 was due to the positive impact on increased digestibility and nutrient absorption in the digestive tract and increase of nitrogen retention materials (Schneitz *et al.*, 1998). Previous investigations indicated that under optimum condition of the digestion and absorption of nutrients for synthesis of thyroid hormones in poultry diet, thyroid gland is more successful in T4 production than T3 production (McNabb *et al.*, 1985; McNichols and McNabb, 1987).

Level of growth hormone directly led to the growth of bones, muscles and other body tissues and indirectly improved bird's growth by stimulating the production and secretion of somatomedin such as Insulin-Like growth factor 1 (IGF-1) especially from the liver tissue (Huybrechts *et al.*, 1985). Biological traits such as growth hormone secretion are influenced by environmental factors. Since growth hormone gene expression is the first stage in the process of protein synthesis, therefore, molecular studies in this stage of protein synthesis could provide useful information about the effect of different factors on growth

hormone synthesis. Hence, effect of two feed additives (thyme extract and probiotic) on growth hormone gene expression was investigated in this study.

The result showed that the thyme extract in dilutions used in this study has no effect on growth hormone gene expression, whereas, growth hormone gene expression was influenced by probiotic usage.

According to the studies carried out by other researchers, probiotic usage, which improved digestion and absorption of nutrients in birds, could play an important role on the biological factors associated with growth (Mountzouris *et al.*, 2010; Schneitz *et al.*, 1998). T4 is considered as an effective factor on bird's growth (Mcnabb and King, 1993; Lazar, 1993). It seems that in this study significant increase in T4 concentration, relate to growth hormone gene expression. Because the result of various studies indicated that thyrotropin- releasing hormone which stimulates production and secretion of thyroid- stimulating hormone from pituitary, and increases production and secretion of T4, could increase growth hormone production (Harvey and Baidwan, 1989; Harvey and Scanes, 1985; Perez *et al.*, 1987; Scanes *et al.*, 1986).

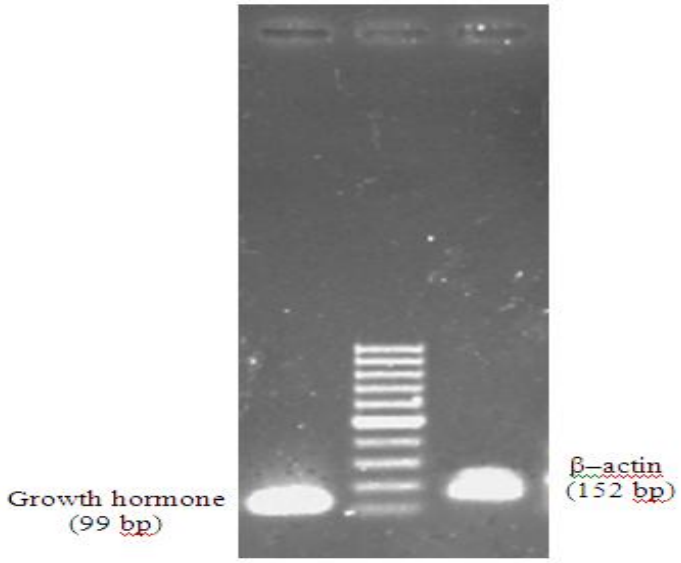

Figure 1: Product of Real-Time qPCR on agarose gel (2%)

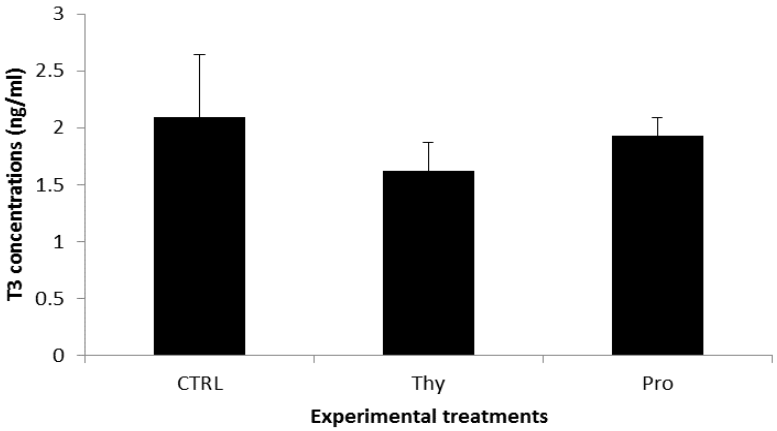

Figure 2: Effect of treatments on serum levels of T3 (mean ± SD).
Means with different superscripts differ significantly (P<0.05).
CTRL: Control group was fed a commercial diet.
Thy: birds were fed control diet containing thyme extract.
PRO: birds were fed control diet + probiotic in drink water

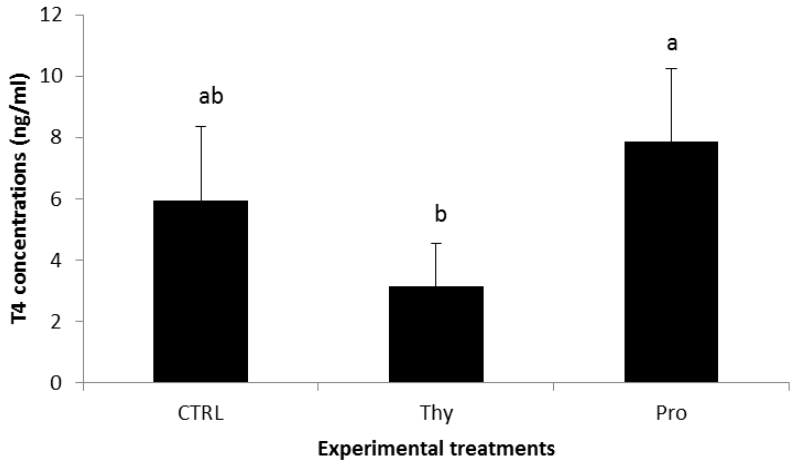

Figure 3: Effect of treatments on serum levels of T4 (mean ± SD). Means with different superscripts differ significantly (P<0.05). CTRL: Control group was fed a commercial diet. Thy: birds were fed control diet containing thyme extract. PRO: birds were fed control diet + probiotic in drink water

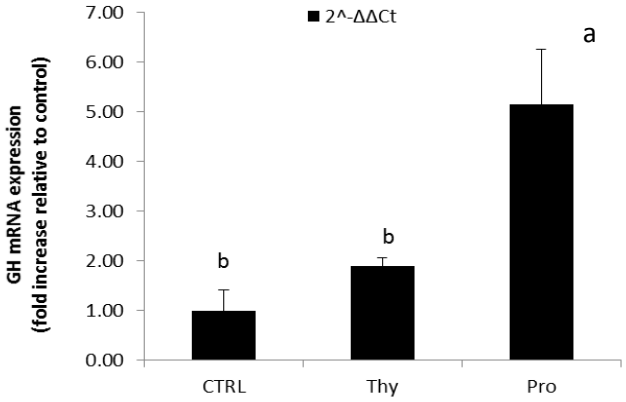

Figure 4: Result of the relative GH gene expression data (mean ± SD) using Real- Time qPCR according to 2-ΔΔct method. Means with different superscripts differ significantly (P<0.05) CTRL: Control group was fed a commercial diet. Thy: birds were fed control diet containing thyme extract. PRO: birds were fed control diet + probiotic in drink water

Mechanism of biological effect of probiotic has not been documented thoroughly (Chichlowski, 2007; Mountzoris *et al.*, 2010) and unfortunately, there is no report available on direct effect of probiotic on growth hormone gene expression in birds. Because there are limited reports about biological impact of dietary factors and probiotic on growth performance, effect of thyme extract and probiotic on growth hormone gene expression and on secretion level of thyroid hormones, more extensive research is required.

Conclusion

This experiment indicated that dietary supplementation of probiotic may influence the biological growth promoters such as T3 & T4 concentrations and GH gene expression in broiler chickens and thyme extract had no effect on these biological parameters.

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