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Effect of mealworm on GBP4L gene expression in the spleen tissue of Ross broiler chickens

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Abstract

Objective

The mealworm (*Tenebrio molitor*) can be applied as both a protein supplement and a prebiotic in poultry diets. It has been shown that its application not only prepares environmental benefits and reduces production costs in poultry farms but also improves poultry health. Therefore, the aim of

this investigation was to measure the relative expression of the GBP4L gene in the spleen tissue of Ross broiler chickens under the effect of dietary mealworm supplementation.

Materials and methods

For this experiment, 20 broiler chicks were randomly divided into two groups of 10 birds each and fed with mealworm as a dietary additive. At the time of slaughter, spleen tissue samples were gathered. Total RNA was extracted applying a total RNA extraction kit. After RNA extraction and purification, the quantity and quality of the RNA were evaluated applying agarose gel electrophoresis and NanoDrop analysis. Complementary DNA (cDNA) was synthesized from the total RNA. To evaluate the relative expression of the target gene (GBP4L) and the reference gene (GAPDH), Real-Time PCR was carried out. Data analysis was carried out applying Excel and Prism software.

Results

The extracted total RNA showed clear and intact 18S and 28S rRNA bands. The RNA exhibited reasonable concentration and was free from protein and alcohol contamination. The amplification and melting curves were standard, and the presence of single bands in gel electrophoresis affirmed the specificity of the reactions. Gene expression analysis indicated that the addition of mealworm to the broiler diet had a meaningful effect ($P < 0.05$) on increasing GBP4L gene expression in spleen tissue compared to the control group.

Conclusions

Based on the results of this investigation and comparison with previous research, it can be concluded that dietary supplementation with mealworm can alter the relative expression of GBP4L in the spleen tissue of broiler chickens. This modulation may enhance immune function and improve resistance to heat stress in broilers. Therefore, GBP4L may serve as a key modulator in improving immune responses, paving the way for further research with larger sample sizes and under diverse physiological and environmental situations.

Keywords: broiler chickens, GBP4L gene, heat stress, immune response, *Tenebrio molitor*

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Introduction

Livestock production, particularly the rearing of domestic chickens, plays a meaningful socio-economic role in supporting the livelihoods of communities in low-income regions of Africa and Asia (Mohamadinejad et al., 2024). Among avian species, domestic chickens (Gallus gallus domesticus) are the most extensively distributed worldwide, largely due to their short generation intervals, rapid growth rate, and remarkable adaptability across diverse agro-ecological zones (Mohammadifar & Mohammadabadi, 2018; Khabiri et al., 2025). These birds serve as an essential source of high-quality animal protein and represent a primary income-generating asset for many rural households in developing countries. In fact, chickens are the most commonly kept livestock species globally, primarily because of their low input requirements and quick reproductive cycle (Mohammadabadi et al., 2010; Mohammadifar & Mohammadabadi, 2018; Mohammadabadi et al., 2024). The popularity and resilience of indigenous and village chicken breeds can be attributed to their possession of valuable traits, containing natural resistance to common diseases, tolerance to environmental stressors, and the capability to thrive on low-quality and locally available feed resources (Shahdadnejad et al., 2016; Khabiri et al., 2023). These adaptive advantages make them ideal for low-input, smallholder production systems that dominate many rural economies. Furthermore, chickens also contribute to food security, women's empowerment, and poverty alleviation, highlighting their multifaceted role in rural development. Guanylate-binding proteins (GBPs) are a large family of interferon (IFN)-inducible GTPases with molecular weights ranging from approximately 65 to 67 kDa. These proteins play a critical role in innate immunity against intracellular pathogens (Li et al., 2017). GBPs bind to GTP and hydrolyze it into GDP and GMP. Structurally, they comprise a globular N-terminal GTP-binding domain, a central helical domain, and a C-terminal helical domain (Olszewski et al., 2006). To date, seven human GBP genes—GBP1 through GBP7—have been identified, all exhibiting a high degree of sequence homology and located on chromosome 1q22.2. In mice, eleven GBP genes (GBP1 to GBP11) have been announced, organized into two gene clusters located on chromosomes 3H1 and 5E5, respectively (Degrandi et al., 2007; Shenoy et al., 2007; Kim et al., 2011). Guanosine-binding protein 4-like (GBP4L) is a member of the GBP family, and recent investigations have begun to elucidate its biological significance. For instance, Feng et al. (2021) demonstrated that GBP4L plays a meaningful role in host defense against bacterial infections. Similarly, Kim et al. (2016) announced that GBPs function as central hubs within the interferon-stimulated gene (ISG) network, mediating essential immune defense mechanisms like immune regulation, inhibition of tumor cell proliferation, and eradication of microorganisms. In avian species, the GBP4L gene is located on chromosome 12. Based on Hamidi et al. (2021), IFNs act as immunoregulatory cytokines that enhance tissue repair through heat stress, modulate

inflammatory responses, and boost the function of immune cells. Based on these roles, it is hypothesized that GBP4L may play a critical role in the inflammatory response related to heat stress. Heat stress influences chromatin accessibility through numerous mechanisms. For example, Xu et al. (2022) announced that heat-induced changes in chromatin structure and accessibility in human K562 cells closely resemble those occurring through the transition from the G1/S to the G2/M phases of the cell cycle. Furthermore, Buenrostro et al. (2013) demonstrated that ATAC-seq is a powerful method for investigating heat stress responses in avian models, as it enables the identification of open chromatin regions. Further investigations by Xu et al. (2022) and Zhu et al. (2023) revealed that these open chromatin regions often contain key regulatory elements like promoters and enhancers. The identification of these regions prepares valuable insight into the regulatory mechanisms of gene expression under heat stress and facilitates the discovery of critical genes involved in the stress response. Mealworm (Tenebrio molitor) has emerged as a promising alternative protein source that can be incorporated into poultry diets without adversely affecting bird growth performance. In an investigation investigating the effects of locally generated mealworm powder supplementation on broiler chickens, parameters like feed intake, body weight gain, feed conversion ratio (FCR), carcass yield, antibody titer, and mortality were evaluated. The results indicated that increasing the dietary inclusion of powdered mealworm cake from 0.1% to 0.3% led to a meaningful ($P<0.05$) improvement in both weight gain and feed conversion efficiency. Notably, the highest carcass yield was recorded in the group receiving 0.3% mealworm powder supplementation (Hussain et al., 2017). These results were further corroborated by Hall et al. (2018), who announced that containing mealworm in broiler diets at levels up to 10% had no detrimental effects on weight gain, feed intake, or FCR. In addition to performance traits, mealworm supplementation also appears to influence broiler physiology. For instance, Ballitoc and Sun (2013) found that diets containing 1% mealworm improved the length of the small intestine while reducing abdominal fat deposition. Similarly, Bellezza Oddon et al. (2021) demonstrated that feeding broilers with 5% live mealworm positively impacted the relative weight of the spleen. Selaledi et al. (2021) also announced improvements in breast meat quality in indigenous Bushveld chickens supplemented with 5% mealworm powder, containing enhanced pH and texture. However, higher inclusion levels (10% and 15%) of mealworm powder were related to negative impacts on certain meat quality traits like improved cooking loss (Ballitoc & Sun, 2013; Bellezza Oddon et al., 2021; Selaledi et al., 2021). Additional adverse effects announced include a reduction in caecal *E. coli* counts following supplementation with 5% yellow mealworm (Sedgh-Gooya et al., 2021). Furthermore, Bovera et al. (2015) observed that broilers fed diets containing 29.7% yellow mealworms exhibited better FCR compared to those fed diets

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containing 44.7% soybean meal. In a subsequent investigation, Bovera et al. (2016) managed a comprehensive assessment of the full replacement of soybean meal with mealworm larvae in broiler diets, centralizing on growth performance, nutrient digestibility, carcass characteristics, and meat quality. Their results showed no meaningful differences in performance, carcass traits, or meat quality among the treatment groups. However, FCR was significantly improved ($P<0.05$) in birds receiving mealworm-based diets. In comparison to the control group (fed with soybean meal), broilers fed mealworm as a sole protein source exhibited significantly more developed gastrointestinal tracts, enlarged spleens, and improved lengths of the intestines, ileum, and caecum ($P<0.05$). Interestingly, the lowest albumin-to-globulin ratio was recorded in the mealworm-fed group, suggesting enhanced disease resistance and immune responsiveness. This improvement may be attributed to the prebiotic properties of chitin and other bioactive compounds naturally exist in insects. Furthermore, biochemical analyses revealed that the levels of aspartate aminotransferase (AST) in the mealworm group remained within the normal range for broilers and were not accompanied by improves in gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH), or creatine kinase—denoting no cellular damage in hepatic or muscle tissues. These results collectively support the feasibility of applying mealworm larvae as a unique, effective protein source in broiler diets (Bovera et al., 2016). Therefore, the objective of this investigation was to investigate the effect of mealworm supplementation as an alternative protein source on the expression pattern of the GBP4L (Guanosine-binding protein 4-like) gene in the spleen tissue of Ross broiler chickens.

Materials and methods

To measure the relative expression level of the *GBP4L* gene, spleen tissue samples were gathered from 16 broiler chickens (Ross strain) at the time of slaughter. For this purpose, 80 broiler chickens were assigned to a completely randomized design with two groups, four replicates per group, and 10 chickens per replicate. The birds were fed diets supplemented with mealworms. The experimental groups were basal diet and basal diet + 0.5% mealworm (freely fed). At the end of the 42-day experimental period, the chickens were slaughtered, and spleen samples were gathered for analysis. Through the experimental period, vaccination was carried out against Newcastle and Gumboro disease strains. Total RNA was extracted from the spleen tissue applying a non-column Total RNA Extraction Kit (DNazist Co., Iran) with slight modifications. After extraction and purification, the quantity and quality of the RNA were evaluated by electrophoresis on a 2% agarose gel applying 1X TBE buffer (for 50 minutes at 94 volts) and a Nanodrop spectrophotometer.

Complementary DNA (cDNA) synthesis was carried out following the manufacturer's protocol (Parestous, Mashhad, Iran). Since the target gene sequences were available in the gene bank, primers for *GBP4L* and *GAPDH* were designed based on sequences retrieved from the Ensembl gene bank (http://www.ensembl.org) applying Primer3Plus, NUPACK, and NCBI tools (Table 1). Primer synthesis was carried out by Sinaclon Co.

Table 1. Sequences of primers applied for *GBP4L* and *GAPDH* genes

Gene	Accession Number	Size of PCR Product (bp)	Primer Sequence (5'→3')
GBP4L	XM_015292901	187	Fwd: ACGCCTGGATCTTCACACTG
			Rev: CAGTCCTCGGCCTCATCTTC
GAPDH	NM_204305.1	132	Fwd: GAACATCATCCCAGCGTCCA
			Rev: CGGCAGGTCAGGTCAACAAC

For the Real-time PCR reaction, a specific Real-time PCR master mix, distilled deionized water (ddw), and specific primers for both the target and reference genes were applied under identical situations. The reaction mixture consisted of 7.5 µL of Cyber Green Master Mix, 1.5 µL of forward and reverse primers, 1 µL of cDNA, and 15 µL of dH2O. The thermal cycling conditions are shown in Table 2. Amplification and melting curves were generated thereafter.

To analyze the Relative Real-time PCR data, Rotor-Gene Q Series Software was applied to calculate PCR efficiency and the quantification cycle (Cq). The results were statistically analyzed applying GraphPad Prism 8 software. One-way ANOVA was applied to evaluate differences in gene expression between treatment and control groups, and significance levels were identified by p-values. A p-value of less than 0.05 ($P < 0.05$) was attended statistically meaningful in all experiments. Charts were plotted applying mean ± standard error of the mean (Mean ± S.E.M.) for each group.

Table 2. Real-time PCR thermal program

Step Completed	Number of Cycles	Temperature (°C)	Duration
Initial denaturation	1	95	15 min
Denaturation (each cycle)	35	95	20 sec
Annealing		63	30 sec
Synthesis		72	20 sec
Final synthesis	1	72	8 min

Results and discussion

Since ribosomal RNAs (rRNAs) constitute approximately 70% of the total RNA content in a cell, the presence of sharp and distinct rRNA bands on an agarose gel is indicative of high-quality RNA extraction. In contrast, the presence of degraded rRNA bands suggests potential degradation of other RNA species and reflects poor RNA quality. Additionally, in RNA extracted from eukaryotic samples, the intensity of the 28S rRNA band should be approximately twice that of the 18S rRNA band (a 2:1 ratio). In the current investigation, the total extracted RNA demonstrated clear and intact 18S and 28S rRNA bands, denoting good RNA integrity (Figure 1). To evaluate RNA quantity, absorbance readings were taken at wavelengths of 230, 260, and 280 nm. The results showed RNA with acceptable concentration and no contamination from proteins or alcohol. To evaluate the synthesized cDNA, agarose gel electrophoresis was carried out. The results showed a single specific band of 132 bp, affirming successful cDNA synthesis for *GAPDH* (Figure 2). Based on the results from Rotor-Gene Q Series software, amplification curves for *GBP4L* and the reference gene began to rise between cycles 23 and 32, entering the exponential phase. Subsequently, the reactions progressed through the linear phase and finally reached the threshold phase. Melting curves were analyzed through the reaction to ensure specific amplification of the genes. The samples exhibited a single peak at 82°C, denoting specific product formation. The electrophoresis results for *GBP4L* further affirmed the results of the melting curves, with the presence of a single band, signifying specificity of the amplification reaction (Figure 2).

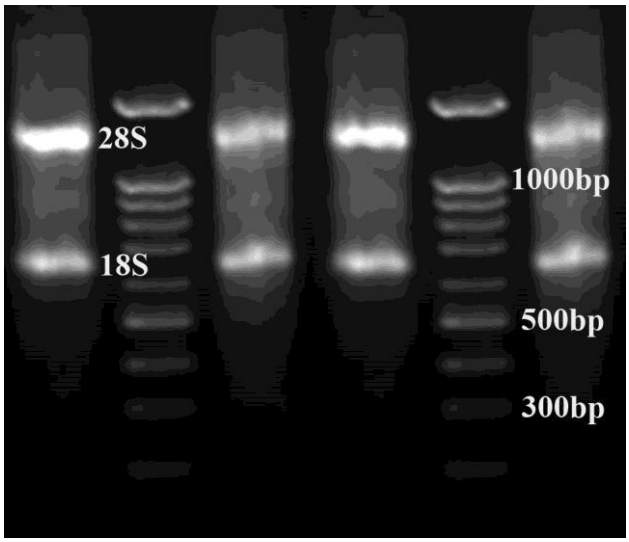

Figure 1. Some samples of extracted RNA on 2% agarose gel

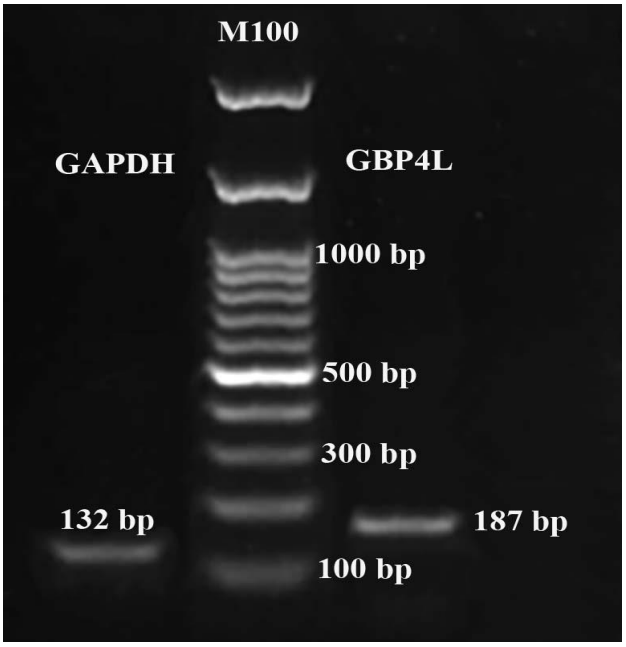

Figure 2. Electrophoresis of PCR products applying *GAPDH* and *GBP4L* primers to evaluate synthesized cDNA. The band size for *GAPDH* is 132 bp, and for *GBP4L* is 187 bp. M100 shows the molecular size marker

In spleen tissue, the inclusion of mealworm in the diet had a meaningful effect ($P < 0.05$) on increasing the expression of the *GBP4L* gene compared to the control group (Figure 3). Nowadays, the application of mealworm (T. molitor) in poultry diets not only supplies part of the needed protein but also helps reduce production costs and improve bird health, while having positive environmental effects. In fact, in 2016, mealworm was approved as a food component in South Korea. Additionally, mealworm has been introduced as an alternative protein source in broiler diets and can be included without negative effects on their growth (Abdel-Hafeez et al., 2017). Mealworms are rich in protein and essential nutrients and likely improve overall nutritional status and reduce stress levels in broilers, potentially leading to improved expression of *GBP4L*. By examining the interaction between these dietary components and relative *GBP4L* expression, we can better understand the holistic impact of nutrition on broiler welfare and performance. Nutrition influences health, welfare, and production through complex interactions of environmental and epigenetic factors, which ultimately lead to exchanges in relative gene expression. Furthermore, a deeper understanding of the complex interactions between nutrition, environment, and gene expression helps improve poultry health, welfare, and productivity. For example, impacts on growth and neurological function affect multiple aspects of animal production containing growth, metabolism, and welfare. Epigenetic mechanisms, which play a key role in these responses, partly function through exchanges in gene expression.

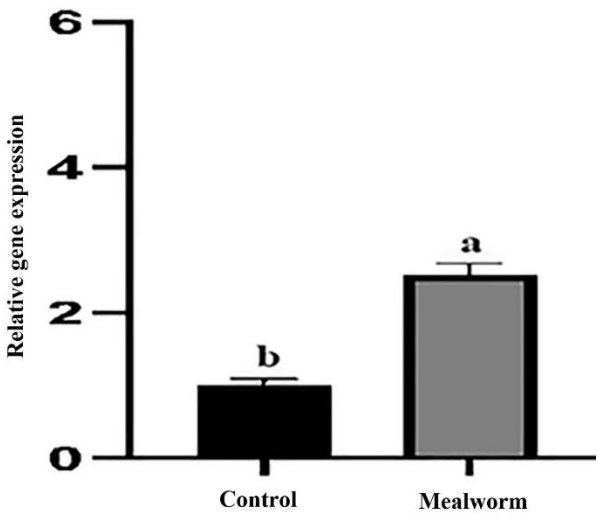

Figure 3. Effect of mealworm supplementation on the relative expression of the *GBP4L* gene in spleen tissue (Groups labeled with different letters are significantly different; P < 0.05)

Furthermore, the spleen's association with immune system function and effects of heat stress highlights its importance in poultry production. On the other hand, researchers have shown that Earth's temperature is rising daily, and this heat and humidity are highly detrimental and damaging to the poultry industry. Since birds lack sweat glands, they cannot efficiently dissipate heat, making them vulnerable to heat stress, which creates meaningful damage. Additionally, selective breeding to improve growth rate and improve feed efficiency has reduced stress tolerance in poultry. Furthermore, when poultry are exposed to high temperatures, egg production, feed intake, fertility rate, and hatchability decline (Ayo et al., 2011; Tang et al., 2020; Kumar et al., 2021; Fathi et al., 2022). Therefore, researchers worldwide are attempting to investigate the factors affecting stress in poultry and are seeking numerous strategies to reduce stress. On the other hand, numerous investigations have shown that the *GBP4L* gene affects heat stress in poultry (Kim et al., 2016; Hamidi et al., 2021). Guo et al. (2021) demonstrated that *GBP4L* plays a critical role in stress-induced immunosuppressive networks. Zhang et al. (2025) applied ATAC-seq technology to investigate the effect of *GBP4L* on chromatin spatial structure, clarifying *GBP4L*'s role in the heat stress response and how immune responses are regulated by heat stress. They showed that *GBP4L* decreases levels of pro-inflammatory and anti-inflammatory cytokines in HD11 cells. They also demonstrated that *GBP4L* regulates the expression of *CCNB3* and *P21*, thereby reducing the number of HD11 cells in the G1 phase and increasing the number in the S phase, which promotes cell cycle progression and improves proliferation. They hypothesized that

GBP4L modulates immune responses by regulating macrophage proliferation. Furthermore, they showed that *GBP4L* improves *Bcl2* expression and decreases *Fas* and *FasL* expression, thereby reducing apoptosis of HD11 cells. Thus, they demonstrated that overexpression of *GBP4L* suppresses heat stress-induced SP9 activation and potentially reduces heat stress-related effects on immune system function in poultry by regulating it. These researchers also announced that *GBP4L* significantly improves immune indices of the spleen and bursa of Fabricius under heat stress in chicks. This gene also reduces spleen damage created by heat stress, highlighting its vital role in maintaining immune homeostasis and protecting immune organs. They believe that *GBP4L* helps chicks cope with heat stress by enhancing spleen function and activating immune cells. Results by Schat (2022) have shown that the bursa of Fabricius is essential and critical for B-cell growth, suggesting that *GBP4L* may improve environmental situations for improved antibody production capacity. Therefore, *GBP4L* has the potential to play a prominent and important role in enhancing immune system performance in poultry. Numerous investigations (Aydin and Hatipoglu, 2024; Baumgard and Rhoads, 2013; Wang et al., 2024) have demonstrated that *GBP4L* reverses heat stress-induced improves in glucose (GLU), alanine aminotransferase (ALT), aspartate transaminase (AST), and total cholesterol (TC), as well as decreases in serum cholinesterase (CHE), alkaline phosphatase (ALP), and triglycerides (TG) in chickens. This indicates that *GBP4L* mitigates the negative effects of heat stress on energy metabolism, liver function, and lipid metabolism. In the current investigation, mealworm supplementation in the diet had a meaningful effect ($P < 0.05$) on increasing the relative expression of the *GBP4L* gene in spleen tissue compared to the control group. It appears that mealworm inclusion in broiler diets can potentially improve *GBP4L* relative gene expression in the spleen. This approach may lead to improved immune function, enhanced capability to cope with heat stress, and ultimately better overall performance in broilers, highlighting the importance of nutritional strategies in poultry management to optimize animal welfare and productivity. These results emphasize the potential of *GBP4L* as an important factor in boosting immune system performance in poultry and open avenues for new research in this area.

Conclusions: The show investigation examined the effect of mealworm supplementation in poultry diets on the relative expression of the *GBP4L* gene in spleen tissue. Results showed that adding mealworm to broiler diets significantly ($P < 0.05$) improved relative *GBP4L* gene expression in spleen tissue. Overall, it can be concluded that mealworm supplementation in broiler diets alters the relative expression of *GBP4L* in spleen tissue. Considering the critical role of this gene in numerous physiological mechanisms containing immunity and heat stress response, further complementary investigations and understanding of the underlying mechanisms for

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modulating relative *GBP4L* expression in target tissues can justify the application of mealworm supplementation.

Author contributions

Conceptualization and design: M.M. and M.A.; Data acquisition: M.M. and A.K.; Data analysis: H. K., O.B., O.O.B., O.K. and O.N.; Data interpretation: M.M., V.A. and V.S.; Manuscript drafting: M.M.; Critical revision of the manuscript: M.M., S.U. and O.B.; Final approval of the manuscript: M.M., O.K. and O.N.; Writing—original draft preparation: M.M., V.A. and V.S.; Writing-review and editing, supervision: M.M. All authors have read and approved the final version of the manuscript.

Data availability statement

The corresponding author will provide the data upon request.

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Ethical considerations

The **current investigation** was **managed** at the Shahid Bahonar University of Kerman, Iran and was approved by the Institutional Animal Care and Use Committee Protocol #IR22979001 **based on** the guidelines of the Iranian Council of Animal Care (1995).

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Conflict of interest

The authors declare that there are no conflicts of interest related to this investigation.

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اثر میلورم بر بیان ژن *GBP4L* در بافت طحال جوجه‌های گوشتی سویه راس

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چکیده

هدف: کرم میلورم (*Tenebrio molitor*) می‌تواند هم به عنوان مکمل پروتئینی و هم به عنوان یک پری‌بیوتیک در جیره طیور استفاده شود. نشان داده شده که استفاده از آن هم فواید زیست محیطی دارد و هم باعث کاهش هزینه های تولید در مرغداری ها

شده و سلامت طیور را افزایش می‌دهد. لذا، هدف این پژوهش اندازه گیری بیان نسبی ژن *GBP4L* در طحال جوجه‌های گوشتی سویه راس تحت اثر افزودن میلورم به جیره بود.

مواد و روش‌ها: برای انجام این آزمایش ۲۰ قطعه جوجه گوشتی در قالب طرح کاملاً تصادفی با ۲ گروه، و ۱۰ قطعه جوجه در هر گروه تحت تغذیه با میلورم به‌صورت افزودنی مطالعه شدند و هنگام کشتار از بافت طحال آنها نمونه‌برداری شد. سپس RNA کل با استفاده از کیت استخراج Total RNA استخراج شد. پس از استخراج و تخلیص RNA، کمیت و کیفیت RNA استخراج‌شده با الکتروفورز روی ژل آگارز و روش نانودراپ مورد بررسی قرار گرفت. از روی RNA کل cDNA ساخته شد. برای ارزیابی بیان نسبی ژن هدف (*GBP4L*) و ژن مرجع (GAPDH) از روش Real-time PCR استفاده شد و نتایج به‌وسیله نرم‌افزارهای Excel و Prism بررسی شد.

نتایج: در مطالعه حاضر، RNA کل استخراج‌شده دارای باندهای rRNA 18S و rRNA 28S واضح و کاملاً سالم بود. نتایج نشان‌دهنده RNA با غلظت معقول و فاقد آلودگی پروتئینی و الکلی بودند. استاندارد بودن منحنی‌های تکثیر ژن و منحنی‌های ذوب و وجود تک باند در نتایج حاصل از ژل الکتروفورز نشان‌دهنده اختصاصی عمل کردن واکنش‌ها بود. نتایج بیان ژن نشان داد که افزودن میلورم به جیره غذایی جوجه گوشتی تأثیر معنی‌داری ($P < 0.05$) بر افزایش بیان ژن GBP4L در بافت طحال در مقایسه با گروه کنترل داشت.

نتیجه‌گیری: بر اساس یافته‌های این پژوهش و مقایسه با نتایج سایر پژوهش‌ها می‌توان نتیجه گرفت که با افزودن میلورم به جیره جوجه‌های گوشتی، می‌توان بیان نسبی GBP4L را در بافت طحال تغییر داد و با این تغییر باعث بهبود سیستم ایمنی جوجه های گوشتی شد و آن‌ها را در مقابل استرس گرمایی مقاوم تر نمود. لذا، می توان پیشنهاد داد که GBP4L پتانسیل این را دارد که به عنوان یک مداخله کننده مهم در بهبود سیستم ایمنی نقش ایفا کند و راه را برای انجام پژوهش‌های جدید با نمونه‌های بیشتر و با در نظر گرفتن شرایط فیزیولوژیکی و محیطی متفاوت هموار سازد.

کلمات کلیدی: استرس گرمایی، پاسخ ایمنی، جوجه‌های گوشتی، ژن *GBP4L*، *Tenebrio molitor*

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