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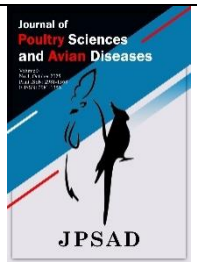

Effects of Oak Acorn on Performance and Gene Expression of Hypothalamus Tissue in Broiler Chickens

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A B S T R A C T

Oak fruit is high in energy and, therefore, can be used as a substitute for corn in poultry diets. However, one limitation of oak fruit is its high level of antinutritional compounds (tannins). This study investigated the effects of corn replacement with oak acorns on performance traits and the mRNA levels of hypothalamic genes in broiler chickens. For this purpose, a total of 264 one-day-old broiler chickens were randomly assigned to three experimental treatments (0, 15, and 20 % oak acorn). Body weight gain, feed intake, and feed conversion were calculated on a pen basis at 21 and 42 days of age. The results showed that body weight gain and feed conversion were significantly affected by treatments at both 21 and 42 days of age; however, no significant difference was observed in feed intake among the treatments. At the age of 21 days, a significant difference in weight gain was observed among the three treatments, whereas this difference was not significant for the treatments with oak acorn on day 42. However, the lowest weight gain was observed in the 20% oak acorn diet at both ages. Feed conversion was significantly higher in 20% of the oak acorn treatment relative to the control group on day 42, while feed conversion was not affected by treatments at the age of 21 days. In addition, the mRNA levels of NPY, AgRP, and Ghrelin were significantly downregulated in the hypothalamus tissue of broilers fed diets containing oak acorn. At the age of 21 days, the expression levels of NPY showed a significant decrease in the hypothalamus tissue of broilers fed with 15% and 20% oak corn diets, while this decrease was significant for the AgRp and Ghrelin genes on day 42. In conclusion, these results suggest that replacing 15% and 20% of corn with oak acorn can negatively affect the performance and hypothalamic gene expression of broiler chickens.

Keywords: *Gene expression, Hypothalamus, Oak acorn, Broiler*

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1 Introduction

Oak is the most abundant and important tree in western Iran, particularly in the Zagros region. Oak fruit is rich in carbohydrates, and its metabolizable energy (ME) is estimated at 3,300 kcal per kg; thus, it can be considered an important source of energy in poultry diets. Additionally, its crude protein, fat, and nitrogen-free extract were 6.1%, 6.8%, and 66.4%, respectively (1). No adverse effects were observed in broilers fed green oak fruit in North Africa (2). Additionally, replacing one-third of the corn in the diet of broiler chickens with oak acorns had no significant effect on performance traits (3). However, it should be noted that oak fruit contains large amounts of antinutritional compounds (tannins), which limit its consumption in livestock and poultry diets. Tannins, a class of secondary metabolites in plants, are a group of high-molecular-weight polyphenolic substances (4). The antinutritional properties of tannins in farm animal nutrition include reducing feed intake, growth rate, nutrient digestibility, and the production and activity of enzymes, as well as the availability of minerals and vitamins (5-8), which ultimately lead to a decrease in the nutritional value and energy of foods (9). Tannins have also been reported to show antioxidant, immunostimulatory, and antibacterial properties (12-14).

Furthermore, tannins affect the activity of organs such as the intestines, liver, and pancreas (10). Of course, the effect of tannins depends on the characteristics (body weight gain, feed intake, and nutritional efficiency), type of tannin in food, tannin concentration, animal factors (species, age, and level of production), length of test period, protein level, and its source (6). In general, tannin levels in food are inversely related to palatability, voluntary intake, digestibility, and nitrogen retention in animals (11, 12). Therefore, the antinutritional effects of oak are intensified by increasing its level in the diet. Numerous studies have confirmed the decreasing effect of dietary tannins on feed intake. Tannins form a complex with salivary proteins rich in Proline, as well as proteins that cover the oral cavity, which reduces appetite and food intake by creating an astringent taste (5). Appetite, which is a perfectly regulated phenomenon, is controlled by hormones and neurotransmitters released from various parts of the body. These compounds affect appetite by modulating factors such as hunger, satiety, and gut motility. The central nervous system and peripheral regulators are the two main mechanisms that regulate appetite, with the hypothalamus playing a vital role in centrally regulating appetite (13). The central nervous system regulates food intake by two types of neurotransmitters (NPY/AgRP and POMC/CART) produced in the arcuate nucleus of the hypothalamus. The NPY/AGRP and POMC/CART neurons have opposite effects on food intake, metabolism, and body mass, with the former stimulating food intake (orexigenic) and anabolic, and the latter inhibiting food intake (anorexigenic) and catabolic properties. In addition to the arcuate nucleus, another nucleus in the hypothalamus called the paraventricular nucleus, modulates food intake and energy expenditure through signals received from the arcuate nucleus (14). This area of the hypothalamus also plays an important role in regulating food intake by producing corticotropin-releasing hormone (CRH). The CRH, like POMC/CART, exhibits potent anorexic properties, leading to decreased appetite in humans (15). Today, the effect of phenolic compounds, such as tannins, has been confirmed on the expression of many genes, including digestive enzymes and immune system proteins, in humans and domestic animals. Considering the role of hypothalamic hormones in regulating appetite and the impact of tannins on feeding intake, it is necessary to investigate the effect of tannin levels on the expression of hypothalamic genes. Therefore, this study aimed to investigate performance traits and mRNA changes in feed-regulating peptides like Neuropeptide Y (NPY), Pro-opiomelanocortin (POMC), Agouti-associated protein (AgRP), Corticotropin-releasing hormone (CRH), Melanocortin receptor 4 (MCR-4), Melanocortin receptor 5 (MCR-5), Cocaine-and amphetamine-regulated transcript (CART), and Ghrelin in the hypothalamus tissue of broilers fed with two levels of 15% and 20% oak acorn in the diet.

2 Materials and methods

The Institutional Animal Care Committee of the University of Yasouj approved all procedures of the current study. A total of 264 Cobb 500 one-day-old broiler chickens were assigned in a completely randomized design with three treatments and four repeats, totaling 22 chickens per pen: 1) control feed, without oak acorn (n= 88), 2) 15% raw oak acorn (n= 88) and 3) 20% raw oak acorn (n= 88). Broiler chickens were randomly assigned to each treatment in pens with ad libitum access to fresh water and diet. All treatments were based on corn and soybean, but they differed in oak acorn content. The diets were formulated according to the recommended nutritional values by the NRC (22) for broilers, using UFFDA software (Table 1). During the experimental period, water and feed were provided freely for the broiler chickens. The weight of broilers was measured in

groups at 1, 21, and 42 days of age, and the weight gain was calculated as the difference in weight between the beginning and end of each period.

Table 1. Ingredients and nutrient composition of experimental diets

Ingredient (%)	Recommended starter	Recommended finisher
Corn1	56.04	61.89
Soybean meal	37.17	31.24
Calcium carbonate	1.25	1.36
Dicalcium phosphate	1.62	1.15
Vegetable oil	2.86	3.46
Vitamin premix2	0.25	0.25
Mineral premix3	0.25	0.25
Salt	0.42	0.32
DL-Methionine	0.14	0.06
Lysine	-	0.02
Nutrient composition		
ME (Kcal/kg)	2950	3050
Crude protein (%)	21.20	19.06
Calcium (%)	0.93	0.86
Available phosphorus (%)	0.42	0.33
Sodium (%)	0.18	0.14
Methionine (%)	0.46	0.36
Lysine (%)	1.01	0.95

In experimental treatments, diet's corn was replaced by oak acorn at 15% and 20%, respectively.

Vitamin premix provided per kilogram of diet: vitamin A, 18,000 IU; vitamin D_3, 4,000 IU; vitamin E, 72 mg; vitamin K_3, 4 mg; vitamin B_1, 3.55 mg; vitamin B_2, 13.2 mg; vitamin B_6, 5.88 mg; vitamin B_9, 2 mg; vitamin B_{12}, 0.03 mg; calcium pantothenate, 19.6 mg; Niacin, 59.4 mg and choline chloride, 1g

Mineral premix provided per kilogram of diet: manganese, 65 mg; zinc, 55 mg; iron, 50 mg; copper, 8 mg; iodine, 1.9 mg and selenium, 0.4 mg.

Table 2. Primers properties

Gene	GenBank (accession no)	Primer sequences (5'-3')	Product Size (bp)
NPY	M87294	F: TGCTGACTTTCGCCTTGTCG R: GTGATGAGGTTGATGTAGTGCC	148
AgRP	NM_001031457	F: GGAACCGCAGGCATTGTC R: GTAGCAGAAGGCGTTGAAGAA	163
Ghrelin	AB075215	F: CCTTGGGACAGAAACTGCTC R: CACCAATTTCAAAAGGAACG	203
CRH	NM_001123031	F: CTCCCTGGACCTGACTTTCC R: TGTTGCTGTGGGCTTGCT	86
POMC	NM_001031098	F: CGCTACGGCGGCTTCA R: TCTTGTAGGCGCTTTTGACGAT	88
MCR-4	XM426042	F: ACACTCCAGCCTCTCCATTTCT R: TGTTCATAGCAGCCTCCCGA	101
MCR-5	AB012868	F: TCCATTCTTCCTCCATCTCATCC R: CTTCCTCATTTCCTGGCTACG	157
CART	BI394769	F: CCGCACTACGAGAAGAAG R: AGGCACTTGAGAAGAAAGG	146
β-actin	NM_205518	F: CTGTGCCCATCTATGAAGGCTA R: ATTTCTCTCTCGGCTGTGGTG	139

On days 21 and 42, 6 birds/treatments were randomly selected and their hypothalamus was immediately isolated according to the chicken brain atlas (16) after slaughter. The hypothalamus samples were stored in liquid nitrogen for RNA extraction. Total RNA was extracted from hypothalamic tissue using the RNX-Plus solution (CinnaGen) according to the manufacturer's instructions. Then, RNA samples were subjected to the DNase treatment.

The quality of extracted RNA was evaluated by agarose gel electrophoresis, while the quantity of RNA was determined using a spectrophotometer. To synthesize the first single-stranded DNA, an AccuPower CycleScript RT Premix (Bioneer) kit was used, and the quality of the obtained cDNA was tested using electrophoresis on a 1% agarose gel. The mRNA expression of hypothalamus genes was quantified using RT-PCR with SYBR Green labeling. Primer sequences (Table 2) for amplification have been described previously by Liu et al. (2012) and Yang et al. (2013). Real-time PCR analysis was performed using a CFX96 Real-time PCR System (Bio-Rad, USA). Each RT-reaction served as a template in a 10 µL PCR reaction containing four pmol of each primer and SYBR green master mix (*HotTaq EvaGreen qPCR kit*, Cinnagen). The mRNA levels of hypothalamic genes were normalized to the β-actin gene as a reference. Real-time PCR results were analyzed using REST, version 2.0.13, to compare the differences in mRNA levels between the two groups. REST is a software that can compare two experimental groups with more than 16 data points in a sample and 16 points per group. Data were analyzed using the General Linear Model (GLM) procedure in SAS software, and significant differences among treatments were investigated using Duncan's multiple range tests at a 0.05 probability level.

3 Results

Table 1 shows the results of the growth rate, feed intake, and feed conversion ratio of broilers' feed diets containing different levels of oak acorn in different experimental periods. The treatments show significant differences in body weight gain means ($p < 0.01$). At the end of the starter period (1 to 21 days of age), the weight gain was significantly higher in chickens fed with the control diets compared to 15% and 20% on the oak acorn treatments ($p<0.01$). Additionally, a significant difference was observed between oak acorn treatments in terms of average weight gain. In the finisher period (22-42 days), no significant differences were observed between oak acorn treatments in terms of weight gain ($p>0.05$). However, weight gain was significantly higher in the control group compared to treatments containing oak acorns ($p < 0.01$). Overall (days 1–42), body weight gain was significantly influenced by levels of dietary oak acorn ($p<0.01$). The highest and lowest overall body weight gains were found in broilers fed diets with and without 20% oak acorn, respectively (Table 3).

Table 3. Effect of dietary oak acorn levels on bodyweight gain feed intake and feed conversion ratio of broilers at different phases of the study

Parameter	Dietary levels of oak acorn			SEM	*p* value
	0	15%	20%		
Body weight gain (g)					
d 1-21	707 [a]	636 [b]	605 [c]	3	0.0001
d 22–42	1234 [a]	1039 [b]	936 [b]	17	0.001
d 1–42	1942 [a]	1675 [b]	1541 [b]	15	0.0001
Feed intake (g)					
d 1-21	919	928	901	32	0.94
d 22–42	2733	2831	2693	52	0.57
d 1–42	3651	3758	3594	79	0.71
Feed conversion ratio					
d 1-21	1.30	1.45	1.49	0.05	0.27
d 22–42	2.23 [b]	2.76a [b]	2.91 [a]	0.07	0.01
d 1–42	1.88 [b]	2.26 [a]	2.34 [a]	0.05	0.008

Based on the results, the feed intake of broilers fed diets containing 0 to 20% oak corn did not differ significantly in the starter, finisher, or entire period of the study. However, the amount of feed intake was the lowest in the 20% oak acorn diet, 15% oak acorn, and control treatments in each period. Significant differences were observed between levels of dietary oak acorn and feed conversion ratio (Table 3). During the various phases, the lowest and highest feed conversion ratios were observed for the control and 20% oak acorn diets, respectively.

The mRNA expression of NPY, AgRP, Ghrelin, CRH, POMC, MCR-4, and MCR-5 genes for two levels of oak

acorn diets (15% and 20% treatments) on days 21 and 42 relative to the control group (without oak acorn) is presented as fold induction in Figure 1. Hypothalamic expression of NPY was significantly decreased in broilers fed with 15% and 20% oak acorn at the age of 21 days (p<0.05). On day 42, a significant reduction in AgRP mRNA levels was observed for treatments containing oak acorns. In addition, Ghrelin expression was downregulated in the hypothalamus tissue of broilers in 15% oak acorn at the age of 42 days.

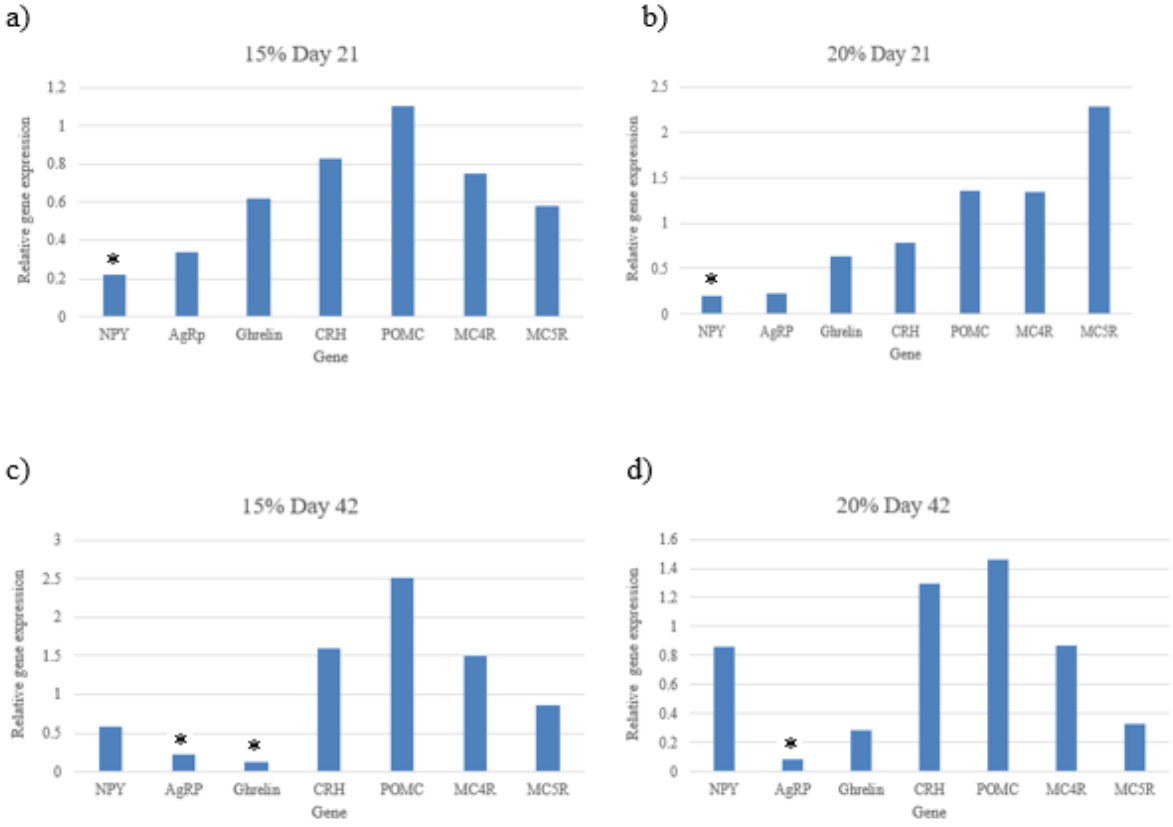

Figure 1. Effect of oak acorn on mRNA levels of neuropeptides in the hypothalamus tissue of broiler chickens.

4 Discussion

Using oak acorns as a substitute for corn in the diet of broilers can reduce feed costs, but their high levels of tannins, an antinutritional compound, can affect the broilers' performance. Therefore, it is necessary to determine the appropriate level of oak acorn in the diet without detrimental effects on broiler performance. The results of this study showed that body weight gain was significantly lower in broilers fed diets containing oak acorns compared to the control group. The observed decrease in broilers' performance can be attributed to the presence of high levels of tannin in oak acorns. These findings, in agreement with previous results, indicate the detrimental effects of oak acorns on broiler performance (24-26). The feed intake and body weight gain were significantly reduced in broiler-fed diets containing 15% and 20% oak acorn (27). Also, the rate of weight gain is significantly reduced in broiler-fed diets with 20% and 25% oak fruit (28). Feeding chicks tannic acid at 0.1% of the diet did not appear to be toxic; however, higher levels (0.5 to 2%) caused a severe reduction in growth rate in a dose-dependent manner (29). In addition, lower body weight was observed in birds fed 33.5% green oak at day 35 of age, but no significant difference was found in final body weight at day 56 of age (2). The inclusion of grape pomace at varying levels (5, 15, and 30 g/kg) in the diet had no significant effect on growth performance or the digestibility of protein and amino acids in broilers (30). Resveratrol, as a polyphenol compound, has been found to improve body weight and average daily gain in broilers subjected to heat stress compared with birds in the control group (31). The average daily weight gain and final live

weight significantly increased (p<0.01) in broiler chickens fed 0.2% grape pomace extract diets compared to the control diet (32). Furthermore, the tannins present in sorghum have also been associated with reduced feed intake, growth rate, protein digestibility, and metabolizable energy, as well as leg abnormalities in chickens (33).

Additionally, the use of tannic acid at levels of 2 to 4% in the diet led to reduced feed intake and egg production in laying hens (34). Midilli *et al*. (2008) reported that the use of different levels (5, 10, 15, and 20%) of oak seed (Quercus cerris) in the Japanese quail diet did not have any negative effects on their performance (35). Feeding the hamsters a diet high in tannin content resulted in weight loss, and this growth inhibition persisted as long as the animals remained on the diet (36). In general, the addition of tannic acid or other forms of phenolic compounds to the diet at a level of 2% has been reported to be toxic (33). However, Sinai and Hooshmand (2016) reported the amount of oak tannin used in this study as 6.07 percent (37). This means that the percentage of dietary tannins in the 15% and 20% oak treatments is 0.91% and 1.21%, respectively.

Based on our results, feed intake in treatments containing oak acorns was not significantly different from that of the control group at various stages of the experiment. Polyphenols and their extracts influence feed intake and energy expenditure by modulating neuropeptides (17) so that their neurological effects strongly depend on polyphenol concentration in the brain. For example, catechin and epicatechin levels were significantly increased in the brain tissue of mice exposed to the polyphenol extract of grape seed for 10 consecutive days compared with mice receiving a single dose (18). Administration of resveratrol to mice also significantly reduces feed intake up to 48 hours after injection (19). In the present study, it appears that the concentrations of dietary tannin (0.91% and 1.21%) are not high enough to affect feed intake in broiler chickens. Generally, the adverse effects of tannins on livestock and poultry performance can be attributed to the inhibition of digestive enzyme activities, particularly pancreatic enzymes (7, 20), and interference with intestinal mucosal glycoproteins (21). The tannins also lead to increased endogenous excretion of essential amino acids, particularly methionine, histidine, and lysine (22). They form indigestible complexes with compounds such as proteins, carbohydrates, and minerals (6). The development of tissue variability in the intestine and liver could be attributed to tannins (10).

The results of this study show that corn replaced by oak acorn alters the expression of some genes (NPY, AgRP, and Ghrelin) affecting feed intake and appetite in the hypothalamus tissue of broilers at the ages of 21 and 42 days. The mRNA levels of NPY, AgRP, and Ghrelin in the hypothalamus tissue of broilers fed with treatments containing oak acorn were found to have decreased in comparison with the control group. In general, the two main parameters, orexigenic and anorexigenic, regulate feeding intake in animals and birds by increasing and decreasing appetite, respectively (23). The orexigenic neurons (NPY/AgRP) have powerful local inhibitory synaptic connections with the anorexigenic neurons (POMC/CART), indicating a strong neuronal circuit that affects appetite (24). Several studies have reported the effects of polyphenol compounds on the expression of neuropeptides that affect feed intake and appetite. For example, resveratrol, as a stilbenoid polyphenol, decreases the expression of NPY and AgRP in a dose-dependent manner (19). At the same time, apigenin increases the expression of POMC and CART in mice hypothalamic cells (25). Administration of Adlay seed water extract to rats fed a high-fat diet had antiobesity effects, as well as a simultaneous decrease in hypothalamic mRNA levels of NPY and LepR genes compared to a normal diet (19).

By releasing the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), the NPY and AgRP neurons mediate most of their orexigenic effects by inhibiting multiple anorexigenic neurons (26). Therefore, in the present study, the decreased expression of NPY and AgRP genes in treatments containing oak acorn reduced their inhibitory role in the release of POMC peptide, leading to increased mRNA levels of this gene. In addition, the melanocortin system coordinates energy balance by regulating both feed intake and energy expenditure in mammals (27) and birds (28). The melanocortin system consists of melanocyte-stimulating hormones (MSHs), adrenocorticotropic hormone (ACTH), a family of five melanocortin receptors (MC1R-MC5R), agouti-related protein (AgRP), and the endogenous melanocortin antagonist agouti (29). However, the POMC is the precursor of the melanocyte-stimulating hormones (MSHs) and adrenocorticotropin (ACTH). In the present study, the lack of a significant effect of oak acorn treatments on mRNA expression levels in the melanocyte system could be attributed to low dietary tannin levels. In other words, adding 15% and 20% oak acorns to the diet of broilers has little effect on the expression of genes that affect appetite suppression (anorexigenic neuropeptides).

Unlike mammals, the Ghrelin hormone plays an anorexigenic role in birds (30) and therefore has opposite effects on appetite and feed intake in poultry. For example, feeding intake was suppressed in a dose-dependent manner by intracerebroventricular administration of Ghrelin in neonatal chicks (18). In this study, the mRNA levels of the Ghrelin gene in the 20% oak acorn treatment group showed a significant decrease compared to the control group. However, in the 15% oak acorn treatment group, Ghrelin expression also showed a decreasing trend. Phenolic compounds can influence the regulation of gene expression by modulating the activity of transcription factors such as NF-κB and AP-1 (31). For example, epigallocatechin gallate (EGCG) in green tea reduces the expression of cytokines, such as IL-6, in intestinal epithelial cells and human brain cells by inhibiting or reducing the binding of the NF-κB transcription factor to DNA (32). In humans, the 5′-flanking region of the Ghrelin gene contains putative binding sites for several transcription factors, such as AP2, PEA-3, Myb, and NF-κB (33). In the present study, the decrease in Ghrelin gene expression in oak-containing diets could be due to the effect of tannin on the activity of transcription factors. Peripherally administration of ferulic acid, a polyphenolic compound, resulted in the reduction of food intake and also in mRNA levels of Ghrelin, melanocortin receptor 3 (MC3R), POMC, and galanin in broiler chicken (34). However, ferulic acid treatment does not affect the expression of NPY, AgRP, MC4R, CART, CRF, GHSR, LEPR, NPY1R, NPY5R, or OREX genes (34).

5 Conclusion

In general, the results of this research showed that the use of oak acorn at the levels of 15% and 20% of the diet in the entire rearing period can have a negative effect on the daily weight gain and food conversion ratio of broiler chickens. In addition, the expression of appetite-related genes in the hypothalamic tissue is affected by the amount of dietary oak acorn. Therefore, it is not recommended to use oak acorn at the levels of 15% and 20% of the diet for the entire rearing period of broiler chickens. Although these negative effects could be prevented by processing the oak acorn to reduce tannin content, additional studies are required on oak processing methods and their effects on reducing tannin content and other oak characteristics.

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Conflict of Interest

The authors declare no competing interests.

Author Contributions

J. St. carried out the experiments; M. M. D. designed, directed, and analyzed the data of the project; A.M. contributed to sample preparation; M. H. contributed to the interpretation of the results. All authors discussed the results and commented on the manuscript.

Data Availability Statement

All data analyzed during this study are included in this article. Any other data are available from the corresponding author upon reasonable request.

Ethical Considerations

All procedures of the current study approved by the Institution Animal Care Committee of Yasouj University.

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