

Effects of different doses of *Foeniculum vulgare* Mill. seeds on growth performance, intestinal and liver histology and gene expression levels in quail

Running title: Effects of fennel on performance, tissue and gene expression in quails

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Effects of different doses of *Foeniculum vulgare* Mill. seeds on growth performance, intestinal and liver histology and gene expression levels in quail

Abstract

This study aimed to determine the effects of ground fennel seed (FS) supplementation to quail feed on growth performance, some slaughter and carcass characteristics, histopathological changes in liver and ileum, and gene expression levels. A single factorial experimental design was used. In total, 160 seven-day-old mixed-sex Japanese quails (*Coturnix coturnix japonica*) were randomly assigned to four groups of four replicates each. Groups were fed with 0, 1, 2, and 4% FS-supplemented basal diet (control, FS1, FS2, FS4), respectively. Study results showed that FS supplementation did not affect growth performance. Intestinal villus height and villus width decreased significantly in female quails with FS supplementation. Crypt depth increased significantly in FS2 and FS4 males, while it decreased in FS2 females. FS supplementation significantly reduced fatty liver grades in females. The peroxisome proliferator-activated receptor alpha (PPAR α) gene level was significantly increased in FS4 females compared to control group, FS1 and FS2 females and males, and FS4 males only. As a result, FS, a phytoestrogenic plant, has shown different effects depending on sex. The increase in PPAR α gene level in FS4 females suggests that FS supplementation affects lipid metabolism in a sex-dependent manner and that the hepatoprotective effect of PPAR α may increase as the amount of FS supplementation increases. Considering its positive effect on the grade of fatty liver and PPAR α gene level, it was determined that FS supplementation at 4% can be used in female quail feeds to protect animal health and support functional food production.

Keywords: Carcass characteristics; Fatty liver; Lipid metabolism; PPAR α ; Quail; Villus morphology; SREBF1 gene.

Study contribution

In poultry feeding, various phytogetic plants are used as feed additives to increase product quality, extend shelf life, and protect animal health and therefore public health by providing functional food to people who consume animal products. This study examined the performance parameters, some carcass and slaughter characteristics, histopathologic changes in liver and ileum, and the expression level of PPAR α and sterol regulatory element-binding transcription factor 1 (SREBF1) genes involved in lipid metabolism by adding fennel seed (*Foeniculum vulgare* Mill) to quail feed at different percentages. The promising potential of fennel seed in preventing fatty liver disease has been demonstrated both by reducing fatty liver in histopathologic examination in a sex-dependent manner and by increasing the PPAR α gene level. In addition, the hepatoprotective effect of PPAR α increased as the amount of fennel increased.

Introduction

Fennel (*Foeniculum vulgare* Mill.), belonging to the Apiaceae family, is a medicinal plant known and used by humans since ancient times.⁽¹⁾ The main component (60–70%) of fennel essential oil is trans-anethole.^(2, 3) Previous studies show that prolonged use of fennel is not harmful to animals, with no serious side effects reported.⁽⁴⁾

Fennel is a valuable plant with potential health benefits due to its diverse phytochemical and nutrient composition, including volatile compounds, flavonoids, phenolic compounds, essential oil, and amino acids.^(4, 5) In addition, it has antioxidant, anti-inflammatory, antimicrobial, and estrogenic effects.^(4, 6–8) İpçak et al.⁽⁹⁾ found that the anti-inflammatory interleukin-10 (IL-10) gene was significantly increased in male broilers fed with encapsulated feed supplemented with 100 mg fennel essential oil compared to the control group. This study demonstrated that fennel treatment in obese rats led to

significant reduction in both hepatic malondialdehyde (MDA) and myeloperoxidase (MPO) levels, as well as continuous decrease in body weight throughout the treatment period.⁽¹⁰⁾ In some studies, the inclusion of fennel seed in poultry diets has been reported to have no significant effect on body weight.^(11–13) On the other hand, some studies have shown that fennel supplementation can lead to an increase in body weight.^(9, 14, 15) Similar inconsistencies have been observed in findings related to feed intake and feed conversion ratio. For instance, while some researchers reported that fennel consumption did not significantly affect feed intake^(11, 13, 15) or feed conversion ratio,^(13, 14) others indicated that fennel supplementation improved feed conversion.^(9, 15)

In a study investigating the effects of fennel on intestinal morphology, improvements were observed in the structural dimensions of the duodenum, jejunum, and ileum in broilers fed with fennel oil. Specifically, a significant increase in muscular layer thickness was reported in the duodenum and jejunum, while a notable increase in muscular layer thickness was observed in the jejunum and ileum.⁽⁹⁾

Broiler chickens fed with fennel powder supplementation showed significant increase in the height and depth of the villus and the villus/crypt ratio in the jejunum.⁽¹⁶⁾ Regarding the effect of fennel on the liver, quails fed a diet containing 1% ground fennel seeds exhibited decreased liver MDA levels and glutathione peroxidase (GPx) activity, while vitamin C levels increased, indicating effective protection against oxidative stress by reducing lipid peroxidation and improving quail efficiency and resistance.⁽³⁾ Al-Atheem et al.⁽¹⁶⁾ reported increased activity of antioxidant enzymes such as superoxide dismutase (SOD) and GPx, which constitute the main line of defense against free radicals, along with a significant decrease in malondialdehyde levels in the blood serum of broiler chickens fed with fennel.

Fennel essential oil was also shown to counteract reductions in superoxide dismutase, catalase, and glutathione activities in mice, inhibit the increased malondialdehyde content in the liver, and prevent genotoxicity and oxidative stress.⁽¹⁷⁾ It was reported that thirteen compounds isolated from the methanolic extract of fennel were tested for human liver microsomal cytochrome P450 3A4 (CYP3A4) inhibition, with 5-methoxypsoralen (5-MOP) showing the most potent, time-dependent inhibitory effect.⁽¹⁸⁾

Hepatic lipid metabolism is regulated by transcription factors such as PPAR α and sterol regulatory element-binding transcription factor 1 (SREBF1). Peroxisome proliferator-activated receptor alpha is a member of the nuclear receptor superfamily and is overexpressed in mammalian organs such as the liver where a lot of fatty acid oxidation occurs.⁽¹⁹⁾ Salem et al.⁽²⁰⁾ reported that fennel extract provided improvement in adult female rats with fatty liver disease and that the promising therapeutic potential of fennel extract against fatty liver disease may be due to its hepatoprotective activities, hypolipidemic effects, and anti-inflammatory characteristics.

This study examined the performance parameters, some carcass and slaughter characteristics, histological changes in liver and ileum, and the expression levels of PPAR α and SREBF1 genes involved in lipid metabolism and related to β -oxidation of fatty acids in quails fed with 0, 1, 2, and 4% ground FS-supplemented feed.

Materials and methods

Ethical statement

The research protocol was approved by Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee with the decision numbered 2022/03-19. Seven-day-old Japanese quails (*Coturnix coturnix japonica*) with an average body weight of 22.87 \pm 0.799–24.29 \pm 0.738 g were used as animal material. A total of 160 mixed-sex

quails were distributed into four groups without causing a statistical difference in terms of average body weight values (each group was divided into four subgroups of 10 quails). The study was conducted at Hatay Mustafa Kemal University Experimental Research, Application and Research Center Alternative Poultry Unit. The nutritional values of corn-based compound feed and the values of the essential oil components of FS are presented in **Table 1**.

Raw nutrient contents of compound feeds were determined according to the methods reported in Association of Official Analytical Chemists. Metabolic energy was calculated using the Turkish Standards Institute formula.⁽²¹⁾ The study groups were divided into control (commercial growth feed +0% FS), FS1 (commercial growth feed +1% ground FS), FS2 (commercial growth feed +2% ground FS), and FS4 (commercial growth feed +4% ground FS). Feeding was done *ad libitum* for 35 days. Quails were placed in equal environmental conditions such as temperature, light, and humidity.

Table 1. The nutrient composition of compound feed and the active ingredients of fennel

Nutrient values of		Active components of fennel seed essential oil			
Mixed feed	Quantity	Active ingredient	%	Active ingredient	%
Dry matter (%)	89.55	Sabinene	0.14	3-cyclohexen-1-ol, 4-methyl-1	0.04
Crude protein (%)	23.7	α -Phellandrene	0.14	Benzene, 1-methoxy-4	25.4
Crude fat (%)	6.17	α -Myrcene	0.07	α -Terpineol	0.04
Crude cellulose (%)	4.1	l-Limonene	5.33	Trans-carveol	0.04
Crude ash (%)	4.96	cis-Ocimene	0.30	2-cyclohexen-1-one,2-methyl-5	0.75
Calcium (%)	0.85	α -Phellandrene	0.03	Trans-anethole	63.16
Phosphorus (%)	0.6	1,8-Cineole	0.19	Phenol, 5-methyl-2	0.05
Sodium (%)	0.2	Benz.1-methylethyl	0.19	Benzaldehyde, 4-methoxy-	1.43
DL-methionine (%)	1.4	Cyclohexanol	0.04	Trans-isodillapiole	0.04
Lysine (%)	0.6	Cyclohexanon	0.06	Anisyl acetone	0.09
Metabolic energy (kcal/kg)	2859.3	Fenchone	1.87	1-Propan.1-(3-ethoxyphenyl)	0.08

Production performance

To determine production performance, the body weights of a total of 160 quails assigned to four groups were recorded weekly using a 0.01 g precision balance (Neck WT20002NF mark®). Starting from the third week of age, the quails were sexed by examining the breast feathers. Those with spotted breast feathers were considered females and those with plain brown breast feathers were considered males.⁽²²⁾ The weekly feed intake of quails in each group was determined. For this purpose, the amount of feed given at the beginning of the week and the amount of feed remaining at the end of the week were weighed and recorded. The feed conversion ratio was calculated using body weight gain and feed intake values (Feed conversion ratio = Feed intake/weight gain).

Carcass characteristics

To determine the carcass characteristics, six females and six males close to the group's average body weight were decapitated from each group. Carcass characteristics were determined by measuring slaughter weight, carcass weight after slaughter (not eviscerated), hollow carcass weight, heart, liver, gizzard, and abdominal fat weights. Carcass yield after slaughter and hollow carcass yield were calculated.⁽²³⁾

Rate calculation:

$$CYAS (\%) = \left(\frac{CWAS}{\text{Slaughter weight}} \right) \times 100$$

CYAS: Carcass yield after slaughter (not eviscerated)

CWAS: Carcass weight after slaughter (not eviscerated)

$$\text{Hollow carcass yeild} (\%) = \left(\frac{\text{Hollow carcass weight}}{\text{Slaughter weight}} \right) \times 100$$

Histological examination

From the left lobe of the liver (approximately 1 cm diameter) and from the distal ileum (about 2 cm long), samples from 48 slaughtered quails (six female and six male quails from each group) were removed and fixed in 10% buffered formalin. Routine tissue process was followed. Tissues were dehydrated in ascending grades of ethanol (70, 80, 90, 96, and 100%), cleared in xylene, embedded in paraffin, sectioned at 5 μ m thickness from each block, deparaffinized in xylene, then passed through a series of 100, 96, 80, and 70% alcohol, respectively, and stained with H&E. After examination under a light microscope (Olympus CX31, Tokyo, Japan), photomicrographs were taken (Olympus DP12, Tokyo, Japan). Histological findings in the liver were evaluated according to the following criteria: Grade 0: histological change in less than 5% of the tissue section. Grade 1: mild; histological changes occur between 5 and 33% of the area. Grade 2: moderate; histological changes occur in 33 to 66% of the area. Grade 3: severe; histological changes occur in more than 66% of the area.^(24, 25)

Histomorphological examination of the ileum was performed as described by Fan et al.⁽²⁶⁾

(Figure 1). A section from the ileum of each animal was examined.



Figure 1. Histological images of the ileum, 100 μm, H&E. 1) Villus height, 2) Villus width, 3) Crypt depth.

Molecular analysis

Total RNA isolation: Total RNA isolation was performed using the TRIzol (TRIzol® Reagent, Ambion, Life Technologies, Carlsbad, CA, USA) method in accordance with the manufacturer's instructions. For this, a 50 mg liver tissue sample was homogenized in 1 mL of TRIzol. A volume of 0.25 mL chloroform was added to the homogenate and quickly overturned for 30 seconds. It was then centrifuged at 4°C, 12 000 × g for 15 min. The supernatant was transferred to a new nuclease-free tube and 0.5 mL isopropanol was added. The samples were rapidly turned upside down for 30 seconds, then centrifuged at 4°C, 12 000 × g for 10 min. A volume of 500 μL of 70% ethanol was added to the RNA pellets and centrifuged at 4°C, 7 500 × g for 5 min. This stage was repeated once. Then, 500 μL of 99% ethanol were added to the pellet and centrifuged at 4°C, 7 500 × g for 5 min. The RNA pellets were then dried and resuspended with nuclease-free water. The isolated nucleic acid concentration and A260/A280 absorbance ratio were determined by a nucleic acid meter (Multiskan GO® Microplate Spectrophotometer, Thermo Fisher Scientific®, USA). Isolated samples were stored at -80°C until use.

cDNA synthesis

The total RNA samples obtained were converted to cDNA using the OneScript Plus cDNA Synthesis Kit (Applied Biological Materials® Inc. (abm), Richmond, Canada, Cat. No. G236). For this, 1 µL dNTP, 1 µL reverse transcriptase, 4 µL 5X RT buffer, 1 µL oligo (dT) primer, 1 µL random primer, 2 µg total RNA, and 2 µL nuclease-free water were added to a total volume of 20 µL according to the manufacturer's instructions and incubated in a thermal cycler at 25°C for 10 min, 50°C for 50 min, and 85°C for 5 min. cDNA samples were stored at -20°C until use.

Real-time quantitative polymerase chain reaction analysis

The expression levels of PPAR α and SREBF1 genes involved in the lipid metabolism pathway were analyzed by real-time quantitative polymerase chain reaction (RT-qPCR). The primer sequences of genes are given in **Table 2**. The glyceraldehyde-3-phosphate dehydrogenase gene was used as the reference gene. The RT-qPCR reaction was performed using the RealQ Plus 2X Master Mix Green (Ampliqon®, Odense, Denmark, Cat. No. A323402) kit. For this, 12.5 µL RealQ Plus 2X Master Mix, 150 nM forward and reverse primers, 100 ng cDNA, and nuclease-free water were added to a total volume of 25 µL according to the manufacturer's instructions. Reaction conditions were: 15 min at 95°C; 40 cycles of 30 s at 95°C and 1 min at 60°C. Each reaction was duplicated. The results of the experiment were given as fold change.⁽²⁷⁾

Table 2. Primer sets used in the study

Gene	Primer sequences	Base pairs (bp)	GenBank ID
PPAR α	F: 5'-GCCTTTCAGTTGGAATGTCACATA-3' R: 5'-CTGCCTTCAACTTGGCCTTCT-3'	78	XM_015870642.2
SREBF1	F: 5'-CGGAAAGCCATCGAGTACATC-3' R: 5'-CCATCCTCAGGCTGAGGTTCT-3'	79	XM_015876643.2
GAPDH	F: 5'-TGAAAGTCGGAGTCAACGGATT-3' R: 5'-CCACTTGGACTTTGCCAGAGA-3'	81	XM_015873412.2

PPAR α : peroxisome proliferator-activated receptor alpha

SREBF1: Sterol regulatory element-binding transcription factor 1

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase gene

Statistical analyses

The study data were analyzed using IBM SPSS Statistics® 22.0 software. One-way analysis of variance (ANOVA) was performed to determine whether growth performance, slaughter characteristics, feed performance, and gut characteristics varied according to the feed content variables. Following the one-way analysis of variance, Duncan's *post hoc* multiple comparison test was used to determine the difference between groups for normally distributed characteristics, and Dunnett's T3 was used for non-normally distributed characteristics. In terms of non-parametric characteristics, the Kruskal–Wallis test was used for group comparison and the Mann–Whitney U test was used in pairs to determine different groups.

Statistical analyses of molecular data were performed using GraphPad Prism version 9.1.1 (GraphPad Software®, San Diego, CA, USA), a licensed commercial software for statistical and graphical analysis. Results are given as mean \pm SEM. P value < 0.05 was considered significant. The expression levels of the genes were normalized according to the expression of the reference gene. The delta-delta cycle threshold method, also known as the $2^{-\Delta\Delta CT}$ method was used to determine the expression levels and the results are presented as fold change.

Results

Effect of FS on the growth performance of quails

Measurements of body weight and feed intake and calculations of feed conversion ratios are summarized in **Table 3**. There was no statistically significant difference between the groups in terms of body weight at baseline, and on days 14, 21, 28, 35, and 42 ($P > 0.05$). There was no statistically significant difference between the groups in terms of the change in feed intake values from day 21 to day 42 ($P > 0.05$). However, between days 14 and 21, there was a statistically significant decrease in feed intake of all groups in which FS supplemented the feed ($P < 0.01$). When the feed conversion ratios of the groups were evaluated, no statistically significant difference was found between the groups except between days 28 and 35 ($P > 0.05$). However, in the period from day 28 to day 35, it was determined that FS-supplemented groups showed a statistically significant increase in feed conversion compared to the control group ($P < 0.01$). Although not statistically significant, the improvement in feed conversion ratio observed in the FS-supplemented groups persisted between days 35 and 42 (**Table 3**).

Table 3. The effect of FS supplementation to broiler quail feeds on performance parameters

Parameters	Groups				SEM	P
	Control	FS1	FS2	FS4		
Body weights (g)						
Onset (7 day)	23.86	23.87	24.29	22.87	0.413	0.660
14 d	50.87	51.59	51.45	49.18	0.877	0.752
21 d	96.10	93.39	96.15	91.33	1.254	0.461
28 d	144.97	141.31	144.75	135.72	1.331	0.051
35 d	180.45	180.85	185.48	179.76	1.556	0.543
42 d	210.58	209.31	212.93	206.05	2.080	0.691
Feed intake (g/day per quail)						
14–21 d	13.80 ^a	12.20 ^{bc}	12.59 ^b	11.48 ^c	0.165	0.002
21–28 d	15.35	14.65	14.79	14.44	0.179	0.356
28–35 d	18.66	17.18	17.16	16.76	0.428	0.446
35–42 d	19.22	21.08	20.12	19.09	0.904	0.852
14–42 d	16.76	16.28	16.16	15.44	0.305	0.521
Feed conversion ratio (g feed intake/g weight gain)						
14–21 d	2.69	2.36	2.47	2.37	0.065	0.298
21–28 d	2.25	2.16	2.16	2.26	0.072	0.927
28–35 d	3.88 ^a	3.11 ^b	2.84 ^b	2.67 ^b	0.109	0.009
35–42 d	5.53	4.97	4.32	4.58	0.565	0.884

14–42 d	3.23	3.10	3.04	2.97	0.094	0.777
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^{abc}: Different letters in the same row indicate different groups ($P < 0.05$). Fennel seed (FS).

Treatment groups: Control, FS1, FS2, and FS4 indicate the supplementation of fennel seed at the rate of 0, 1, 2, and 4% of diet, respectively. Standard error of the mean (SEM).

P-value significance level ($P < 0.05$; $P < 0.01$).

Effect of FS on slaughter and carcass characteristics

Values for carcass parameters are summarized in **Table 4**. There was no statistically significant difference between the groups in terms of mean slaughter, carcass weight after slaughter (not eviscerated), hollow carcass weight, heart and abdominal fat weights, and carcass yield after slaughter (not eviscerated) and hollow carcass yield ($P > 0.05$). FS supplementation significantly increased gizzard weight only in FS4 female quails compared to the control group ($P < 0.05$).

Table 4. Effect of Fennel seed supplementation to broiler quail feeds on slaughter and carcass characteristics

Parameters	Sex	Groups				SEM	P
		Control	FS1	FS2	FS4		
Slaughter weight (g)	Mixed	205.65	205.26	205.85	198.87	3.250	0.848
	Male	183.72	191.37	191.92	185.49	2.511	0.576
	Female	227.58	219.14	219.77	212.25	3.741	0.562
CWAS (g)	Mixed	158.02	156.73	155.67	150.88	2.745	0.809
	Male	139.60	146.51	144.11	139.26	1.975	0.508
	Female	176.43	166.95	167.23	162.50	3.385	0.537
Hollow carcass weight (g)	Mixed	122.51	125.44	125.15	120.83	1.583	0.693
	Male	115.45	121.16	119.33	115.56	1.746	0.586
	Female	129.57	129.73	130.97	126.10	2.174	0.873
Heart weight (g)	Mixed	1.84	1.95	1.87	1.90	0.044	0.805
	Male	1.66	1.84	1.81	1.83	0.063	0.733
	Female	2.01	2.07	1.92	1.97	0.056	0.815
Liver weight (g)	Mixed	5.35	5.79	5.24	5.21	0.268	0.860
	Male	3.65	4.68	4.18	4.15	0.121	0.051
	Female	7.06	6.90	6.30	6.28	0.376	0.833
Gizzard weight (g)	Mixed	3.99	4.43	4.20	4.71	0.118	0.179
	Male	3.60	3.81	3.73	3.98	0.109	0.675
	Female	4.38 ^b	5.04 ^{ab}	4.67 ^{ab}	5.44 ^a	0.130	0.049
	Mixed	1.99	1.65	1.99	1.48	0.117	0.321

Abdominal fat weight (g)	Male	1.50	1.58	1.63	1.13	0.126	0.499
	Female	2.48	1.72	2.36	1.84	0.177	0.361
CYAS (%)	Mixed	76.75	76.36	75.52	75.77	0.221	0.207
	Male	76.00	76.50	75.09	75.04	0.248	0.119
	Female	77.49	76.15	75.95	76.49	0.348	0.428
Hollow carcass yield (%)	Mixed	59.96	61.19	61.00	60.87	0.475	0.803
	Male	62.85	63.25	62.18	62.29	0.308	0.586
	Female	57.06	59.13	59.82	59.44	0.703	0.527

^{abc}: Different letters in the same row indicate different groups ($P < 0.05$). Fennel seed (FS). Treatment groups: Control, FS1, FS2, and FS4 indicate the supplementation of fennel seed at the rate of 0, 1, 2, and 4% of diet, respectively. Standard error of the mean (SEM). P-value significance level ($P < 0.05$). CYAS: Carcass yield after slaughter (not eviscerated). CWAS: Carcass weight after slaughter (not eviscerated).

Effects of FS on histological assessment of ileum

The statistical results of ileum villus height, villus width, and crypt depth in quails fed with FS-supplemented feed are summarized in **Table 5**. Although a decrease in villus height was observed with the FS supplementation, this decrease was statistically significant only between the control group and the FS2 group ($P < 0.05$). The highest villus height was measured in FS2 male quails with 383.21 μm and the lowest was measured in FS4 male quails with 296.85 μm . However, the differences in villus height of male quails were not statistically significant ($P > 0.05$). The highest villus height was 540.90 μm in the control group female quails and the lowest was 226.80 μm in FS2 female quails, and these

differences were statistically significant ($P < 0.01$). FS supplementation to the feed decreased the villus width in female quails compared to the control group ($P < 0.05$), but did not cause a statistical difference in the villus width of male quails ($P > 0.05$). It was determined that there was a statistical increase in crypt depth in FS2 and FS4 male quails ($P < 0.01$) and a statistical decrease in FS2 female quails ($P < 0.01$).

Table 5. Effect of Fennel seed supplementation to broiler quail feeds on ileum villus height, villus width, and crypt depth

Parameters	Sex	Groups				SEM	P
		Control	FS1	FS2	FS4		
Villus height (μm)	Mixed	435.46a	377.61ab	305.00b	353.67ab	14.834	0.028
	Male	330.03	309.45	383.21	296.85	2.677	0.204
	Female	540.90 ^a	445.77 ^b	226.80 ^c	410.49 ^b	4.942	0.001
Villus width (μm)	Mixed	81.33	70.32	63.88	66.69	11.14	0.122
	Male	67.90	73.80	58.50	65.46	3.693	0.502
	Female	94.76 ^a	66.85 ^b	69.25 ^b	67.93 ^b	5.974	0.042
Crypt depth (μm)	Mixed	81.71	94.22	70.53	103.24	14.712	0.114
	Male	56.28 ^b	62.20 ^b	89.28 ^a	96.41 ^a	3.511	0.010
	Female	107.13 ^a	126.25 ^a	51.78 ^b	110.07 ^a	4.472	0.002

a, b, c Different letters in the same row indicate different groups.

Sex-based comparison

Groups	Villus height (μm)	Villus width (μm)	Crypt depth (μm)
Control female	540.90 ^a	94.76	107.13 ^{ab}
Control male	330.03 ^{cd}	67.90	56.28 ^d
FS1 female	445.77 ^b	66.85	126.25 ^a
FS1 male	309.45 ^{cd}	73.80	62.20 ^{cd}
FS2 female	226.80 ^e	69.25	51.78 ^d
FS2 male	383.21 ^{bc}	58.50	89.28 ^{bc}

FS4 female	410.49 ^b	67.93	110.07 ^a
FS4 male	296.85 ^{de}	65.44	96.41 ^{ab}
SEM	9.227	2.548	3.731
P-value	0.001	0.055	0.001

a, b, c, d, e Different letters in the same column indicate different groups ($P < 0.05$). Fennel seed (FS).

Treatment groups: Control, FS1, FS2, and FS4 indicate the supplementation of fennel seed at the rate of 0, 1, 2, and 4% of diet, respectively. Standard error of the mean (SEM). P-value significance level ($P < 0.05$; $P < 0.01$).

Effect of Fennel seed on histological assessment of the liver

The results of fatty liver grades in quails fed with different levels of Fennel seed-supplemented feed are summarized in **Table 6**. While the fatty liver grade was the highest in the control group with 1.50, it was observed that there was a decrease in the fatty liver grades of the quails with FS consumption (FS1 = 0.67 and FS2 = 0.83), and it even decreased to 0.50 in the FS4 group and reached a grade one-third of the control group. However, this decrease did not create a statistical difference between the groups ($P > 0.05$).

Table 6. The effect of Fennel seed supplementation to broiler quail feeds on fatty liver grades

Parameters	Sex	Groups				X ²	P
		Control	FS1	FS2	FS4		
Fatty liver	Mixed	1.5	0.67	0.83	0.5	4.915	0.178
Grade		(0–3)	(0–2)	(0–2)	(0–2)		
	Male	0.33	0.33	0.5	0.17	1.437	0.697
		(0–1)	(0–1)	(0–1)	(0–1)		
	Female	2.67	1	1.17	0.83	12.702	0.005
		(2–3) ^a	(0–2) ^b	(0–2) ^b	(0–2) ^b		

^{ab}: Different letters in the same row indicate different groups.

Sex-based comparison

Groups	Level of fat deposition
Control male	0.33 (0–1) ^b
Control female	2.67 (2–3) ^a
FS1 male	0.33 (0–1) ^{bc}
FS1 female	1 (0–2) ^b
FS2 male	0.5 (0–1) ^{bc}
FS2 female	1.17 (0–2) ^b
FS4 male	0.17 (0–1) ^c
FS4 female	0.83 (0–2) ^{bc}
X ²	25.176
P-value	0.001

^{a, b, c} Different letters in the same column indicate different groups ($P < 0.05$). Fennel seed (FS).

Treatment groups: Control, FS1, FS2, and FS4 indicate the supplementation of fennel seed at the rate of 0, 1, 2, and 4% of diet, respectively. Standard error of the mean (SEM). P-value significance level ($P < 0.01$).

There were statistically significant differences between the groups in terms of fatty liver grades depending on sex. The effect of FS supplementation on the grade of fatty liver in

male quails was found to be statistically insignificant ($P > 0.05$). However, it was determined that FS supplementation to the feed significantly decreased the grade of fatty liver in female quails compared to the control group ($P < 0.01$). The fatty liver grade of the female quails in the control group was determined to be as high as 2.67 (**Figures 2 A and B**). This value was higher than the values determined in the male and female quails of the other groups, and there was a statistically significant difference between the control group female quails and the male and female quails of the other groups in terms of the grade of fatty liver ($P < 0.01$). In the sex-based comparison, the lowest grade of fatty liver was determined in FS4 male quails, and this decrease was found to be statistically significant compared to control male, control female, FS1 female, and FS2 female quails ($P < 0.01$) (**Figures 3–6**).

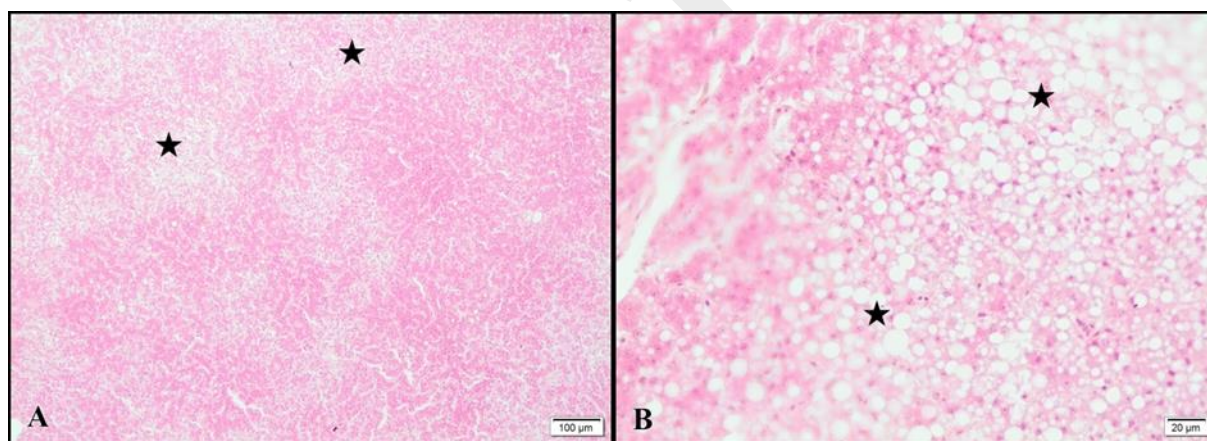


Figure 2. Histological findings observed in the liver, H&E. A) Control female; grade 3 fat vacuoles in the liver (stars), 100 μm . B) Control female; grade 3 fat vacuoles in the liver (stars), 20 μm .

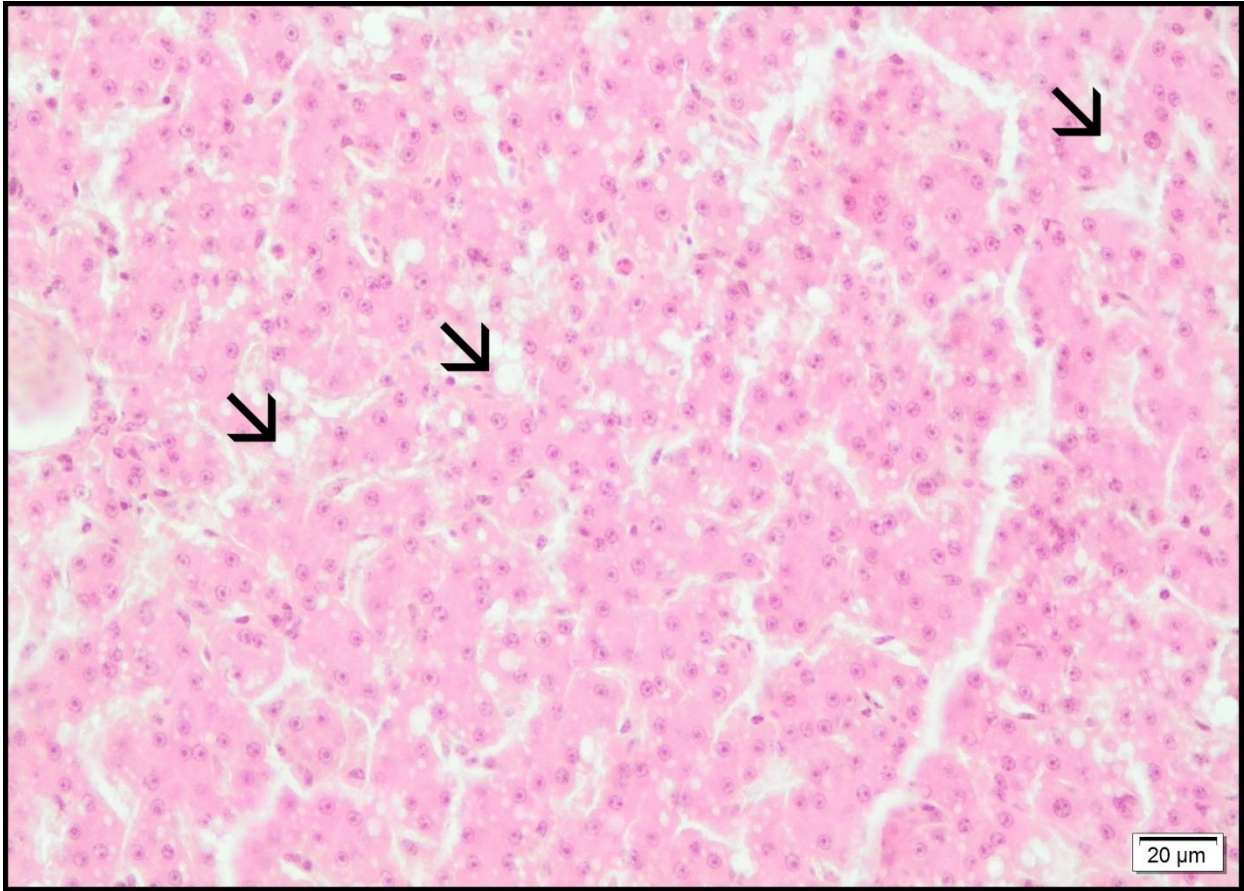


Figure 3. Histological findings observed in the liver of FS2 group females; grade 1 fat vacuoles in hepatocytes (arrows), 20 μm , H&E.

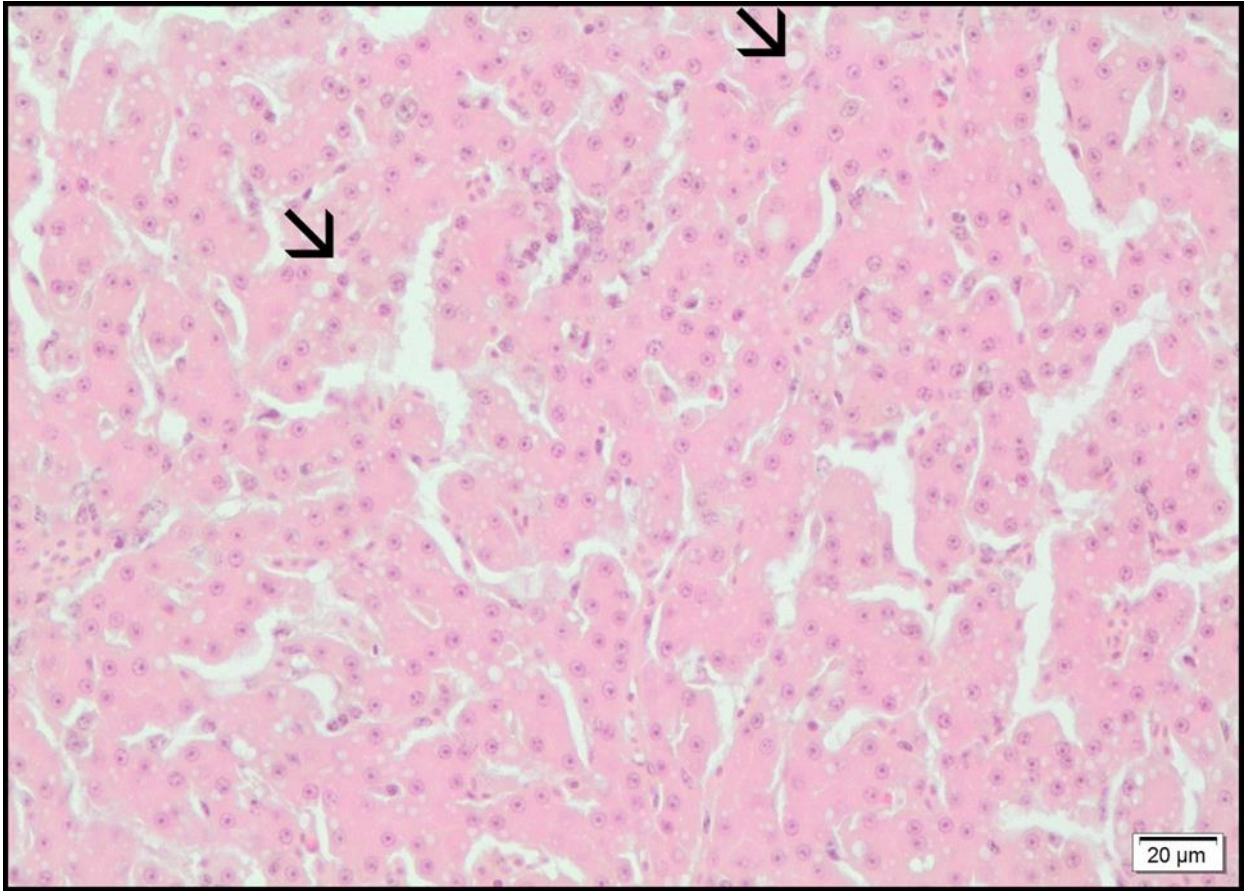


Figure 4. Histological findings observed in the liver of FS4 group females; grade 1 fat vacuoles in hepatocytes (arrows), 20 μm , H&E.

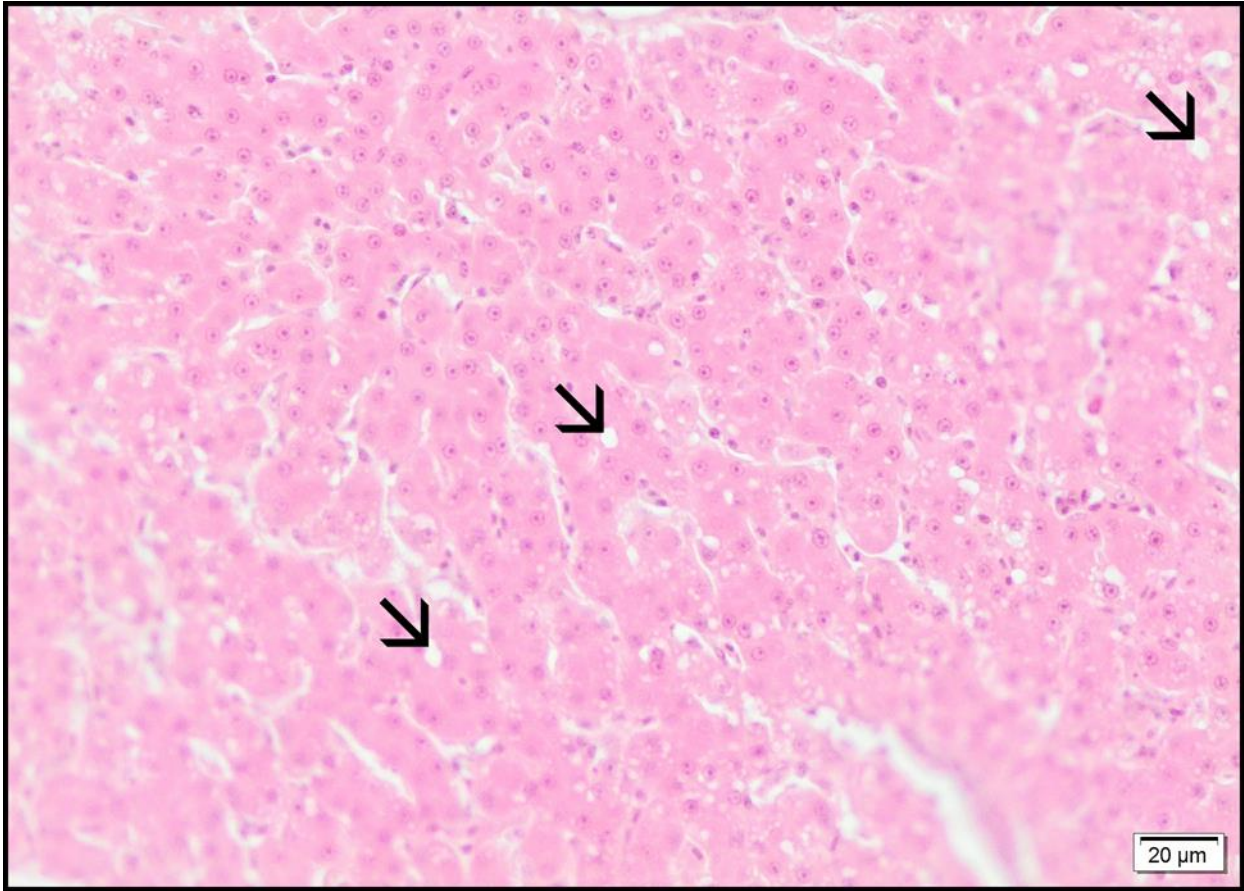


Figure 5. Histological findings observed in the liver of control group males; grade 1 fat vacuoles in the liver (arrows), 20 μm , H&E.

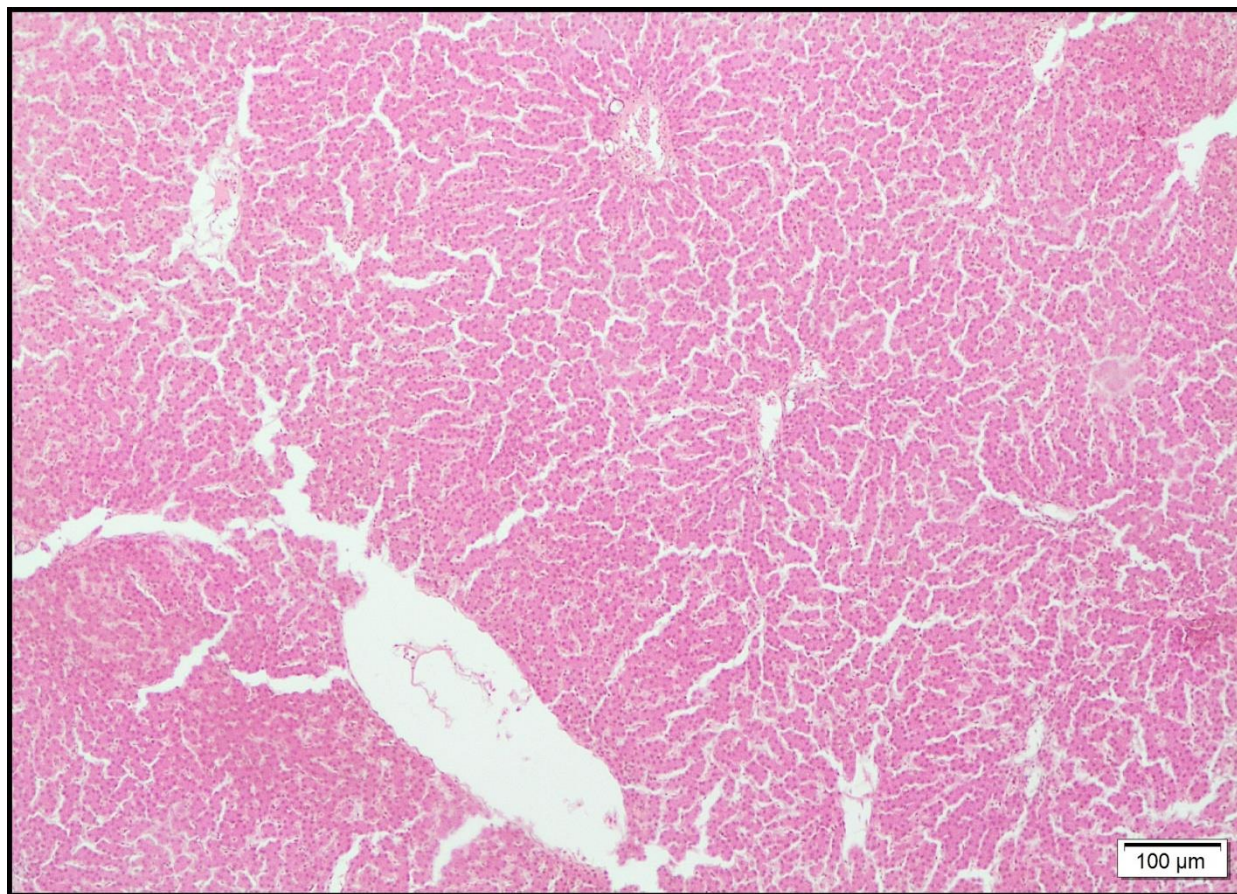


Figure 6. Histological findings observed in the liver of FS4 group males; the liver is normal in terms of fatty liver, 20 μm, H&E.

Effect of Fennel seed on the expression of genes involved in lipid metabolism

The PPAR α and SREBF1 gene expression levels in liver tissue were analyzed to determine whether FS supplementation has an effect on lipid metabolism in quail. While no significant difference was observed in SREBF1 gene level between the groups ($P > 0.05$) (**Figure 7b**), the PPAR α gene level significantly increased ($P < 0.05$) in FS4 female quails compared to control female and control male quails (**Figure 7a**). A significant increase in PPAR α gene level was observed in FS4 female quails compared

to FS1 female, FS1 male, FS2 female, FS2 male, and FS4 male groups ($P < 0.05$) (Figure 7a).

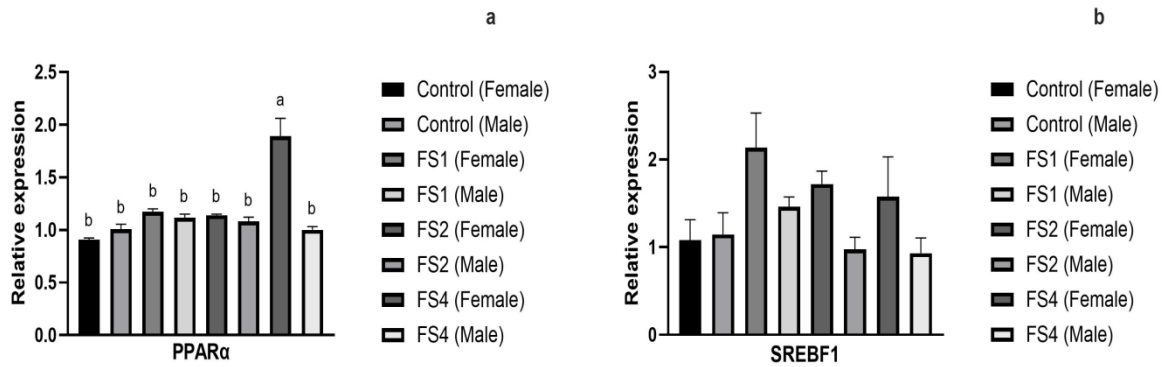


Figure 7. Analysis of relative expression of PPAR α (a) and SREBF1, (b) genes in quail liver tissue by RT-qPCR. One-way ANOVA was used for comparison between groups and Tukey was used as a *post hoc* test. All data were presented as mean \pm SEM. Different superscripts in the same figure represent significant differences. ^{a, b} Different letters above the bars indicate significant differences between groups ($P < 0.05$; $P < 0.01$).

Discussion

FS supplementation to the diet did not affect the body weight compared to the control. Similarly, Liu et al.⁽²⁸⁾ reported that the supplementation of 0.15, 0.30, and 0.45% FS powder did not affect the body weight of broiler chickens. The results of this study showing that FS does not affect body weight in quails are also consistent with the literature.^(11, 12, 29) On the other hand, it is also reported that the supplementation of 1.6 and 3.2% FS powder to the feed did not affect the body weight of broiler chickens under normal conditions but increased the body weight by 3.2% under temperature stress conditions.⁽¹³⁾ Safaei-Cherehh et al.⁽³⁰⁾ observed weight loss in male broiler chickens fed fennel extract (0, 100,

200, 300 and 400 ppm). The results of the present study are contrary to the results of previous studies which reported that feeding with FS supplementation led to an improvement in body weight. Mohammed and Abbas⁽³¹⁾ reported that the addition of different amounts of fennel to feeds resulted in significant improvements in the body weight of chickens. Premavalli and Omprakash⁽¹⁴⁾ stated that the body weight increased significantly at the end of the sixth week in the groups fed with FS powder supplementation at doses of 1 and 1.5%. Similarly, İpçak et al.⁽⁹⁾ reported that the supplementation of fennel seed oil to broiler feeds at 50, 100, 200, and 400 mg/kg significantly increased body weight on days 21 and 42.

In the present study, although there was a significant decrease in feed intake between days 14 and 21 in the FS groups, this decrease was not observed in the other weeks. However, the decrease in feed intake observed in the first weeks with FS supplementation did not affect the feed conversion rate; moreover, it provided a significant increase in the period from day 28 to day 35 compared to the control group. From day 35 until the end of the study, the improvement in feed conversion continued, although not statistically significant. The decrease in feed intake in the first week can be attributed to the very distinctive taste and smell of fennel. Unusual taste or smell can sometimes temporarily reduce the desire to consume the feed in animals and then return to its normal course.

In conclusion, in the present study, FS supplementation did not affect feed intake but improved feed conversion rate, although not statistically significant. Different results were obtained in similar studies. Buğdaycı et al.⁽¹¹⁾ reported that the supplementation of 0.3, 0.6, and 0.9% FS to the feed did not affect the feed intake and feed conversion of quails except for the 0.6% dose. Numerous studies have reported that feed intake or feed

conversion efficiency are not affected.^(13, 14, 28) Mohammed and Abbas⁽³¹⁾ stated that 1, 2, and 3 g/kg fennel supplementation to the feed provided significant improvements in feed efficiency in chickens. The contrasting effects of fennel on growth performance in previous studies may be attributed to differences in fennel storage conditions, feed composition, fennel concentration, application time, or application methods.

The inclusion of FS in the feed did not affect the slaughter and carcass characteristics, except for liver weight in FS1 male quails and gizzard weight in FS4 female quails. Al-Sagan et al.⁽¹³⁾ reported that different doses of FS powder supplementation to feed did not affect carcass characteristics in broiler chickens. Different studies have reported that adding fennel to feed leads to insignificant differences for all carcass characteristics.^(31, 32) It is evident from this study that such phytogetic plants are more effective on animal health rather than growth and yield performance parameters. In studies examining the effects of fennel on intestinal morphology, it has been stated that fennel oil improves villus height, villus width, and crypt depth in ileum morphology that are accepted as indicators of a healthy intestine in broilers⁽⁹⁾ and causes a significant increase in villus height, villus depth, and villus height/depth ratio in the jejunum.⁽¹⁶⁾ Liu et al.⁽²⁸⁾ stated that a dose effect was observed at the addition of 0.30% fennel, which, as an intermediate dose, affected villus height and increased it compared to the control group, while this increase was not observed in the lower and upper doses (0.15 and 0.45%), and crypt depth did not change.

In the present study, it was noted that the villus height and villus width of male quails did not change with FS supplementation, while there was a decrease in both villus height and villus width in female quails. When crypt depth was evaluated, it was determined that dose as well as sex came to the fore. The crypt depth increases as the

amount of fennel consumed in male quails increases. However, the situation is slightly different with FS2 females. It is noteworthy that FS2 female quails have the lowest villus height as well as the least crypt depth. Therefore, this may be either related to the female quail samples belonging to the FS2 group or the number of samples examined in general. As a result, it was observed that these changes in ileum morphology caused by FS did not have a negative effect on the performance parameters of quails.

Although FS was not statistically significant at the rate of 1, 2, and 4% in females and 4% in males, it was observed that when added to the feed, it reduced the amount of fat vacuoles in the liver. Gene expression results also support this. FS affects lipid metabolism in quail liver tissue in a sex-dependent manner in terms of the PPAR α gene but not in terms of the SREBF1 gene. FS supplementation at a 4% level increased PPAR α gene expression level in the liver of female quails compared to control females and control males. This suggests that the effect of fennel on reducing fatty liver may be related to the increase in PPAR α gene levels. PPAR α plays a significant role in the homeostasis of energy and lipid metabolism.⁽³³⁾ PPAR α is known to be highly expressed in chicken liver.⁽¹⁹⁾ Hong et al.⁽³⁴⁾ administered inhaled fennel oil in rats and found that both body fat and visceral fat levels decreased and lipid metabolism improved. However, in this study, no change was observed in the PPAR α gene expression level of the 1 and 2% FS-supplemented groups. These results suggest that the hepatoprotective effect of PPAR α may increase as the amount of FS supplemented in the feed increases in a sex-dependent manner. However, the fact that no significant difference was observed between the groups in the SREBF1 gene level, which is also involved in lipid metabolism, may be related either to the amount of FS supplementation administered or to the fact that fennel does not activate the related pathway. Salem et al.⁽²⁰⁾ reported that fennel extracts improved liver

function and histopathological alterations in adult female rats with fatty liver disease, suggesting that its therapeutic potential may be attributed to hepatoprotective, hypolipidemic, and anti-inflammatory properties. It is considered that more comprehensive molecular-level studies are needed in the future to clarify the biological mechanisms mediating the sex-dependent effects of fennel on the liver.

Conclusions

The study demonstrated that the inclusion of different levels of FS in the diet did not have a significant effect on the growth performance, carcass, and slaughter characteristics of quails. However, FS supplementation was found to induce significant changes in ileum morphology. Additionally, all three levels of FS supplementation significantly reduced liver fat accumulation in female quails. In male quails, only the 4% FS level showed a tendency to reduce the amount of fat vacuoles and fatty change. An increase in PPAR α gene expression was observed depending on FS levels, whereas no significant changes were detected in SREBF1 gene expression. FS has a promising potential in preventing fatty liver disease, demonstrated by the increasing hepatoprotective effect of PPAR α as the amount of FS increased. According to these results, more detailed studies are needed to elucidate the mechanisms underlying the sex-dependent effects of FS on the ileum and liver, and on PPAR α gene expression.

Data availability

All relevant data are within the manuscript and its supporting information files.

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Conflicts of interest

The authors have no conflict of interest to declare in regard to this publication.

Author contributions

Conceptualization and formal analysis: T Cimrin, S Alasahan.

Data curation, investigation, writing-original draft, writing-review, and editing: T Cimrin, S Alasahan, A Eraslan Sakar, T Kutlu.

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