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Influence of flaxseed combined with thyme, rosemary, and sage leaves as fodder additives on antioxidant status in the liver of Japanese quail

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Abstract: The aim of this study was to investigate the influence of a combination of plant supplements with antioxidant properties on the total antioxidant status measured by FRAP and the activity of selected antioxidant enzymes in the livers of Japanese quails. The birds were given feed with the 4% addition of flaxseed combined with a 1% admixture of dried thyme, rosemary, or sage leaves, respectively. The results showed an increased value of the antioxidant potential in the livers of quails fed with a diet that contained the combination of flaxseed and thyme, compared to the control diet and the diet that included flaxseed and rosemary. In all groups given dietary herbal supplements, males and females did not differ in liver antioxidant capacity, whereas in the control group decreased values of these indices for males were found. In the groups fed with the addition of flaxseed and herbs, the activity of glutathione peroxidase and superoxide dismutase was significantly lower than in the control group. There were no differences in the activity of catalase and glutathione S-transferase between the analyzed groups. The use of flaxseed with the addition of thyme, rosemary, and sage leaves had a significant effect on liver antioxidant status in the studied quails.

Key words: Quails, liver, antioxidant enzymes, FRAP, herbs

1. Introduction

The optimal nutrition of the Japanese quail requires various nutrients including antioxidants that are important components of many metabolic pathways (1). The main function of those compounds is to maintain directly or indirectly a balance in the cellular content of reactive oxygen species (ROS). In quail feeding, such a role can be played by additives such as selenium or vitamins that also significantly influence the reproductive ability, adaptation to thermal conditions, and quality of meat (2).

Apart from synthetic additives, the high levels of antioxidant compounds such as phenolic acids, flavonoids, and carotenoids in some herbs or plant seeds (e.g., flax) nowadays have resulted in the growing popularity of such plant supplements in poultry nutrition. Some supplements added to a broiler chicken diet, e.g., the extracts of rosemary, thyme, and oregano, significantly improve meat digestibility and resistance to oxidation (3). The herbs' phenolic acid after absorption from the gastrointestinal tract are transported by the bloodstream to the liver and then conjugated and distributed to other tissues (4). Flaxseed, characterized by a high content of tannin and a number of other phenolic acids and flavonoids, can also significantly influence the composition of fatty acids in the liver of laying hens (5).

In quails and other animals, the liver is the center of biochemical transformations that affect the entire body. As the center of detoxification and synthesis of a number of essential substances, it is particularly vulnerable to sudden increases in the levels of ROS and many other prooxidants. ROS concentrations are controlled by specialized metabolic pathways where the central role is played by superoxide dismutase (SOD), an enzyme that synthesizes hydrogen peroxide, further degraded by catalase (CAT) or glutathione-dependent peroxidase (GSHPx) to water and oxygen (6). This first line of antioxidant defense is supplemented by glutathione S-transferase (GST), which conjugates reactive electrophilic compounds with glutathione and initiates their detoxification (7).

The purpose of this study was to evaluate the effect of herbal supplements, i.e. rosemary, thyme, and sage leaves, in feed enriched with flaxseed on the antioxidant potential and activity of selected antioxidant enzymes in the quail liver.

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2. Material and methods

2.1. Diets and husbandry

During the experimental period, the birds were reared in cages (100 × 60 × 20 cm) while maintaining standard husbandry practices for quails. A total of 240 quails of 23 days old were divided into five groups of 48 specimens each, which were given different diets (Tables 1 and 2). The treatments were finished at 71 days of the quails' lives. For further analysis 16 specimens from each group were randomly selected with equal numbers of females and males.

2.2. Material preparation and biochemical analysis

Immediately after birds' anesthetization with carbon dioxygen, fresh liver samples were homogenized on ice

Table 1. The experimental diets of groups.

Treatment group	Diet
C	Control - without additives
F	Basal diet + 4% flaxseed
FS	Basal diet + 4% flaxseed + 1% sage leaves
FT	Basal diet + 4% flaxseed + 1% thyme
FR	Basal diet + 4% flaxseed + 1% rosemary

Table 2. The detailed composition of the control and basal diets.

Ingredients	Diet	
	Control	Basal
	Amount (%)	
Barley meal	15.00	15.0
Corn meal	25.00	10.0
Soybean meal (46.00% crude protein)	25.00	25.0
Wheat meal	31.76	42.76
Flaxseed	-	4.0
Calcium phosphate	0.70	0.70
Sodium chloride	0.22	0.22
Sodium hydrogen carbonate	0.20	0.20
Limestone	0.80	0.80
L-Lysine HCl	0.29	0.29
DL-Methionine	0.23	0.23
Lutamix DJR premix*	0.50	0.50
Natuphos (BASF)	0.20	0.20
Calculated nutrient content	(g/kg)	(g/kg)
ME (kcal)	2629	2629
Crude protein	200.37	208.76
Crude fiber	39.00	42
Crude fat	22.50	33.61
Lysine	11.70	12.00
Methionine	5.22	5.32
Ca	8.00	8.00
Available P	6.44	6.55

*Vitamin and trace mineral elements (per kg of feed): vitamin E 60.13 mg, vitamin K 2 mg, vitamin B_1 6.15 mg, vitamin B_2 9.78, vitamin B_6 9.13 mg, vitamin B_{12} 40.00 µg, folic acid 3.42 mg, niacin 81.43 mg, biotin 0.40 mg, pantothenic acid 20.73 mg, linoleic acid 5.97 g, choline chloride 1.64 g, magnesium 1.76 g, potassium 7.46 g, manganese 0.127 g, iodine 0.8 mg, copper 14.79 mg.

with ten volumes of 50 mM Tris-Cl buffer (pH 8.1), 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged for 20 min at 4.0 °C and 15,000 rpm. Supernatants were collected and stored at –80 °C for further analysis.

2.2.1. Antioxidant capacity assay in liver

The ferric reducing ability of plasma (FRAP) (8) method was used for antioxidant power assay in the liver tissue homogenates. First, 120 µL of liver homogenates, diluted at 1:3 in deionized water, was transferred to 1 mL of working FRAP reagent preincubated to 37 °C and mixed well. Absorbance was read at 6 min at 593 nm. For calibration ascorbic acid was used in the range of 0.125 mM to 2 mM. Antioxidant status were expressed as ascorbic acid equivalent per gram of wet weight (ww) of liver tissue.

2.2.2. Glutathione peroxidase activity assay

Activity of GSHPx was assayed according to established methods (9). For the assay a RANSEL kit (RANDOX Lab.) was used with cumene hydroperoxide as the substrate. GSHPx activity was defined as the amount of enzyme that oxidizes 1 nmol NADPH per minute per milligram of extracted protein at 37 °C and pH 7.2.

2.2.3. Glutathione-S-transferase activity assay

Twenty microliters of buffer sample diluted in an assay (0.1 M phosphate buffer, 2 mM EDTA, pH 6.5) was transferred to 960 µL of the assay buffer with 10 µL of 100 mM reduced glutathione and 10 µL of 1 mM 1-chloro-2,4-dinitrobenzene (10). After 1 min of lag time, increase of absorbance was monitored for 3 min with 15-s intervals at 25 °C. One unit of GST activity expresses the amount of enzyme that catalyzes the formation of 1 µmol of CDNB-glutathione conjugates per minute per milligram of protein.

2.2.4. Superoxide dismutase activity assay

The SOD activity was determined by ability to inhibit the autoxidation of pyrogallol using the method described by Marklund and Marklund (11). To 960 µL of 50 mM Tris-Cl buffer (pH 8.2) and 1 mM EDTA was added 10 µL of 10 µM bovine erythrocyte catalase solution in 50 mM Tris-Cl (pH 8.2) and 8.5 µL of 24 mM (in 10 mM HCl) pyrogallol solution. Subsequently, 20 µL of appropriately diluted sample was added to the reaction mixture. After 30 s of lag phase an increase of absorbance at 420 nm was monitored for 2 min with 15-s intervals. One unit of SOD was defined as the amount of supernatant that decreases autoxidation of pyrogallol to 50% at pH 8.5 at 25 °C. Activity was expressed as amount of SOD units per milligram of protein.

2.2.5. Catalase activity assay

CAT activity was measured according to established methods (12). To 245 µL of 50 mM phosphate buffer (pH 7.0) and 5 mM H_2O_2 was added 5 µL of appropriately diluted sample and decrease in absorbance at 240 nm was recorded for 2 min following a 15-s lag phase. One unit of enzyme decomposes 1 µmol of H_2O_2 per minute at 25 °C and pH 7.2. Activity was defined as the amount of CAT units per milligram of protein.

2.2.6. Protein concentration assay

The concentration of extracted proteins was assayed by the Bradford method (13).

2.3. Statistical analysis

The obtained data were analyzed statistically with the Statistica 9.0 PL statistical package using the least squares method of the GLM procedure according to the following linear model:

$$Y_{ijk} = \mu + a_i + b_j + e_{ijk},$$

where Y_{ijk} = trait measured; µ = the overall mean; ai = the effect of herbal additives (i = C, F, FT, FR, FS), bj = the effect of sex (j = male, female); and eijk = random error. A detailed comparison of mean least squares for the analyzed groups of quails was performed using Duncan's test.

3. Results

3.1. FRAP in liver tissue of quails

A significantly higher value of FRAP relative to the control group was found in the group of quails receiving a diet with combined flax and thyme ($P < 0.01$) (Table 3). In the liver of quails with a diet supplemented with combined flax and rosemary, a significantly lower ($P < 0.05$) FRAP value compared to the group receiving flax and thyme was found. The rest of the tested fodder mixes did not significantly affect the FRAP values in the livers of quails compared to the control diet group and between other diet groups. Moreover, an occurrence of a significant interaction ($P < 0.05$) was shown between experimental groups and sex in the FRAP values. The roosters in the control group were characterized by a substantially lower FRAP value in relation to all other groups except for hens that were fed with feed containing combined flax and rosemary. In the livers of the hens from this group a significantly lower value of FRAP ($P < 0.01$) was found compared to the roosters that received feed with the addition of only flaxseed and roosters from the group fed with feed that contained a mix of flax and thyme.

3.2. Activity of antioxidant enzymes

Significantly higher levels of SOD activity were found in the control group compared to the groups fed with flax and thyme ($P < 0.01$) and flax and rosemary and sage and flax ($P < 0.05$). A significantly ($P < 0.01$) higher level of activity of GSHPx was found in the control group compared to the group receiving the feed combined with flax alone and the rest of the analyzed herbs. There were no statistically significant differences between the analyzed dietary groups of quails in the activity of CAT and GST.

Table 3. The comparison of FRAP values and antioxidant enzyme activities in livers of the analyzed diet groups of quails.

	Sex	Diet					Total
		C	F	FT	FR	FS	
FRAP mmol/g ww	♂	$1.15^{Bb} \pm 0.12$	$1.65^{A} \pm 0.08$	$1.85^{Aa} \pm 0.08$	$1.57^{ac} \pm 0.10$	$1.51^{ac} \pm 0.12$	1.55 ± 0.06
	♀	$1.62^{ac} \pm 0.08$	$1.52^{ac} \pm 0.15$	$1.66^{A} \pm 0.13$	$1.38^{bc} \pm 0.15$	$1.61^{ac} \pm 0.08$	1.56 ± 0.05
Total		$1.38^{B} \pm 0.09$	1.59 ± 0.08	$1.75^{Aa} \pm 0.08$	$1.48^{b} \pm 0.09$	1.56 ± 0.07	*
GSHPx U/mg protein	♂	1046.19 ± 138.77	657.08 ± 73.44	612.27 ± 70.68	664.00 ± 107.77	670.53 ± 46.13	730.01 ± 47.00
	♀	1224.49 ± 190.09	789.63 ± 74.59	611.75 ± 73.30	659.76 ± 144.53	545.11 ± 85.20	766.15 ± 64.89
Total		$1135.34^{A} \pm 115.99$	$723.35^{B} \pm 53.38$	$612.01^{B} \pm 49.19$	$661.88^{B} \pm 87.09$	$607.82^{B} \pm 49.52$	n.s.
SOD U/mg protein	♂	80.98 ± 7.54	53.72 ± 9.61	40.16 ± 8.69	60.13 ± 9.03	45.47 ± 5.59	56.09 ± 4.16
	♀	65.35 ± 11.81	56.09 ± 8.57	45.80 ± 10.62	50.44 ± 6.14	53.19 ± 9.91	54.17 ± 4.20
Total		$73.16^{Aa} \pm 7.06$	54.90 ± 6.23	$42.98^{B} \pm 6.67$	$55.29^{b} \pm 5.42$	$49.33^{b} \pm 5.59$	n.s.
CAT U/mg protein	♂	179.37 ± 24.89	130.47 ± 32.76	148.99 ± 20.74	184.75 ± 73.91	199.82 ± 43.54	168.68 ± 18.89
	♀	110.42 ± 29.01	134.98 ± 39.56	153.96 ± 25.82	151.88 ± 19.91	211.27 ± 38.36	152.50 ± 14.34
Total		144.89 ± 20.50	132.72 ± 24.82	151.47 ± 16.01	168.31 ± 37.22	205.55 ± 28.07	n.s.
GST U/mg of protein	♂	4.27 ± 0.57	4.71 ± 0.53	3.21 ± 0.25	4.17 ± 0.40	3.81 ± 0.43	4.03 ± 0.21
	♀	5.01 ± 0.55	3.81 ± 0.59	3.78 ± 0.40	4.13 ± 0.31	4.26 ± 0.68	4.20 ± 0.23
Total		4.64 ± 0.39	4.26 ± 0.40	3.49 ± 0.24	4.15 ± 0.24	4.03 ± 0.40	n.s.

Results are expressed as mean ± standard error of mean.
A, B – Different superscripts denote statistically significant differences at $P < 0.01$.
a, b – Different superscripts denote statistically significant differences at $P < 0.05$.
n.s. – Not significant.

4. Discussion

The purpose of this study was to evaluate the effect of herbal supplements, i.e. rosemary, thyme, and sage, in feed enriched with flaxseed on the oxidative potential and antioxidant activity of selected enzymes in quail liver.

Although the high antioxidant potential of herbs rich in polyphenolic compounds usually translates into a hepatoprotective effect (14), inadequate use may be counterproductive and adversely affect the animal's metabolism (15). In this research on quail livers, it was assumed that the impact of antioxidants present in herbal supplements could be measured by the expected increase in FRAP. According to Wojdyło et al. (16), the highest antioxidant capacity measured with FRAP should be observed for rosemary, followed by thyme and then sage. This order results from differences in the levels of fatty acids and flavonoids; for example, *Salvia officinalis* has a significantly higher content of polyphenolic compounds compared to thyme and rosemary, but lower levels of flavonoids. The dried leaves of sage also show a greater diversity in the composition of phenolic acids, as manifested by the presence of p-coumaric acid and neochlorogenic acid, not found in thyme and rosemary. On the other hand, the latter two have clearly higher levels of ferulic and caffeic acids (17).

Differences in the compositions of phenolic acids in the aforementioned herbs may lie behind differences in the responses of the quail liver antioxidant system to each of the analyzed herbal supplements. FRAP increased only in the livers of the flax/thyme group relative to the control and flax/rosemary groups. This somewhat confirms the results of Sengül et al. (18), whereby the blood antioxidant status in Japanese quails increased after the addition of dried thyme to feed. However, Ozcelik et al. (19) demonstrated that the effect of rosemary oil addition to quail feed depends on the dose; at 150 mg/kg it exerts significant antioxidant effects, while at 250 mg/kg it has a prooxidative effect and leads to liver necrosis. This indicates the significance of administration method for the bioavailability of substances found in herbal feed supplements.

Antioxidant potential may also significantly depend on the sex of quails. For example, female rats have higher FRAP in the liver than males (20), which can be explained by the potential effect of female hormones and the differences in the levels of alpha-tocopherol in the liver (21). In this research, this difference was observed only in the control group, while in the other two groups FRAP was similar for males and females. Although there are no data on sex-related differences in quail liver FRAP, such

differences in antioxidant potential are reported for the red blood cells of these birds (22), whereby it was higher in females than males.

In the present study, flaxseed was the primary component of diets in all analyzed groups except the control group. In addition to high levels of antioxidants, flaxseed is also rich in estrogen-like lignans, polyphenols with a strong effect on hormonal balance. Lignans bind to estrogen receptors and so may reduce the effect of endogenous estrogen. In addition, lignans can also lower estrogen levels by inhibiting the activity of aromatase, an enzyme responsible for the conversion of testosterone to estrogen (23). The herbal supplements used in this study may also have a significant effect on estrogen metabolism due their high levels of phenolic acids and flavonoids. For example, rosemary extract increases the rate of hepatic oxidative metabolism of estrogen in mice, thus weakening estrogen activity (24).

In the analyzed quails, only the females from the group supplemented with rosemary and flaxseed showed a decline in FRAP to a level comparable to the males in the control group. Other herbal supplements in this study also contain polyphenols and flavonoids that are potential antagonists to endogenous estrogen (25). Similar FRAP for both sexes in most of the analyzed groups may be related to the effect of substances contained in herbs on the hormonal changes in the tested quails. However, this hypothesis requires further research for determination of various hormones.

4.1. Antioxidant enzymes and the effect of herbal supplements

The effects of phenolic acids vary between individual antioxidant defense enzymes. The expression of GSHPx and SOD genes increases most in response to gallic acid, while acid catalase is most responsive to p-coumaric acid (26). In addition, profiles and levels of phenolic acids in herbal supplements may create synergies manifested by a significant increase in the activities of all antioxidant enzymes; this can also be observed for their antimicrobial activity (27). However, in this study, increased activity of both SOD and GSHPx was only found in the control group, probably a result of moderate oxidative stress manifested by the increased activity of some antioxidant enzymes in the short term.

Moreover, phenolic acids are exogenous antioxidants with an ability to inactivate free radicals and thereby reduce the level of oxidative stress, which may potentially result in a decreased activity of antioxidant enzymes (28). In contrast to SOD, CAT, and GSHPx, polyphenolic substances contained in herbs may be potent inhibitors of GST activity in vitro (29). However, some in vivo studies demonstrated that the addition of these substances increased GST activity in the intestines and kidneys and had no effect on the activity of this enzyme in the liver (30). In this study, the fact that the measured GST activity in quail livers did not change significantly in any group indicates no effect of the used herbal supplements on this element of liver metabolism.

In summary, it seems that the combined herbal additives influenced the antioxidant status in the livers of Japanese quails. Many previous studies reported the beneficial role of these supplements on the typical parameters of quail husbandry, such as reproduction and the quality of eggs and meat. However, the active compounds of supplements may also exert specific effects on metabolic pathways, and in order to predict the nature of these potential changes, it is necessary is extend the monitoring of metabolism with many additional biochemical parameters in the blood or organs of quails. These include the potential changes in the levels of hormones, as the active compounds in herbal additives present in the diet are capable of affecting hormonal metabolism in animals.

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