

Effects of dietary alfalfa flavonoids on the performance, meat quality and lipid oxidation of growing rabbits

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Objective: The present experiment has tested the effect of dietary alfalfa flavonoids (AAF) supplementation on the productive performances, carcass characteristics, meat quality and lipid oxidation of growing rabbits.

Methods: One hundred and sixty crossbred rabbits (42 days old) were divided into four groups of forty animals each and were fed either a control diet (AAF0) or an AAF0 diet supplemented with 400, 800, or 1,200 mg of AAF/kg per diet (AAF4, AAF8, and AAF12, respectively) from weaning to slaughtering (102 days old). Performance data were recorded over a period of 60 days. At the end of the trial, 12 rabbits were slaughtered per group, and the carcass characteristics were recorded. Moreover, the plasma, liver and dorsal muscles were sampled from 12 rabbits/group, and were analyzed for lipid oxidation.

Results: No significant differences were recorded for the performance, carcass characteristics and meat quality traits except for lightness parameter that was lower in the control group. Dietary AAF supplementation significantly ($p < 0.01$) affected the malondialdehyde (MDA) levels of the frozen meat in a dose-related manner, with the lowest value (0.24 mg MDA/kg fresh meat) recorded in the AAF12 group samples.

Conclusion: These findings indicated that the dietary inclusion of AAF in rabbit diets improved muscle oxidation stability with no adverse effects on the growth performance of the animals even if a slight impact on meat lightness color parameter was recorded.

Keywords: Herbal Additives; *Medicago sativa*; Natural Antioxidant; Phytochemicals; Plant Extract

INTRODUCTION

Increasing attention has been paid recently to issues related to the presence of natural biologically active substances in animals feed mixture, which would enrich products of animal origin and at the sometime improve animals' health [1]. Flavonoids are natural substances that are present in vegetables and fruits and are valuable components of animal and human diet. Flavonoids are large group of ubiquitous molecules and possess antioxidant activities; their planar structure, number, and position of their hydroxyl groups, are crucial for free-radical scavenger capacities and the inhibition of free-radical producing enzymes [2]. Alfalfa (*Medicago sativa* L.), which plays an important role among forage crops, due to its high biomass production and good nutritional quality, could be an interesting source of phytochemical compounds. Flavonoids are one of the biologically active ingredients of alfalfa [3]. To date, a number of flavonoids have been identified in various plant parts of alfalfa [3-5]. The most important alfalfa flavonoids (AAF) were the flavones: tricetin and apigenin glycosides [6] and they possess a higher antioxidant activity than the conventional antioxidants such as butylated hydroxytoluene. AAF, coumestrol and apigenin in particular, have shown good antioxidant properties in a variety of low-density lipoprotein oxidation systems,

and to work synergistically with ascorbic acid and other antioxidants [7]. The use of alfalfa and its products in animal feeds as forage is a by now consolidated topic of study [8,9], while only a few studies have been carried out on the use of alfalfa extracts in animal nutrition trials, and these have mainly been conducted on birds [10-12]. AAF with estrogenic effect, was shown to significantly promote growth performance, improve carcass quality, remove free radicals and increase body's antioxidant and enhance immunity in a certain range of additive on livestock [13,14]. Chen et al [15] reported that AAF extraction adding in the diet of Yang Zhou geese aged from 28 to 70 days can improve dietary intake, but shows no adverse effects on slaughter performance, organs and blood biochemical indexes. Lipid oxidation is the main problem in rabbit meat. It is caused by a high polyunsaturated fatty acid content, which can lead to oxidation, and this, in turn, can reduce its shelf life. Generally, little attention has been paid to the effects of a concentrated extract of flavonoids dietary supplementation on growing rabbits in nutritional trials. In light of the above considerations, the objective of this study was to evaluate the potential efficacy of the supplementation of AAF on the performance, carcass traits and lipid oxidation of the plasma, liver and meat of growing rabbits.

MATERIALS AND METHODS

The trial was carried out at the experimental rabbitry of the Department of Agricultural, Forest, and Food Sciences (DISAFA; University of Turin), in Carmagnola, NW Italy. The experimental protocol was designed according to the guidelines of the current European and Italian laws on the care and use of experimental animals (European Directive 86 609/EEC, put into law in Italy with D.L. 116/92). The experimental protocol was approved by the Ethical Committee of the DISAFA of the University of Turin (Italy).

Preparation of the alfalfa extract

A sample of fresh alfalfa (*Medicago sativa* L.) was submitted to a hot water extraction to obtain an extract used later to flavonoids extraction according to Dong et al [10]. Briefly, alfalfa, air-dried for 2 to 3 days to a 15% moisture content, was boiled in hot water at 100°C for 2 h. The obtained liquid solution was then filtered, and the extract was spray-dried and stored until analysis.

High-performance liquid chromatography coupled with photodiode array and tandem mass spectrometry analysis of the alfalfa flavonoids extract

Alfalfa extract was diluted in water at a concentration of 10 mg/mL. This stock solution was diluted in a solution of water/acetonitrile (95/5) at a concentration of 5 mg/mL and analyzed in duplicate with a Shimadzu Nexera X2 system equipped with a photodiode detector SPD-M20A in series to a triple quadruple Shimadzu LCMS-8040 system provided with electrospray ionization (ESI)

source (Shimadzu, Dusseldorf, Germany). Samples were analyzed on an Ascentis Express C18 column (15 cm×2.1 mm, 2.7 µm, Supelco, Bellefonte, PA, USA) using water (containing 0.1% formic acid) and acetonitrile (containing 0.1% formic acid) as mobile phases A and B, respectively. The flow rate was 0.4 mL/min and the column temperature was maintained at 30°C. The gradient program was as follow: 5% B for 3 min, 5% to 15% B in 17 min, 15% to 25% B in 15 min, 25% B for 5 min, 25% to 5% B in 2 min. The total pre-running and post-running time was 47 min. UV spectra were acquired in the 220 to 700 nm wavelength range and the resulting chromatograms were integrated at 338 nm. MS operative conditions were as follows: heat block temperature: 200°C; desolvation line temperature: 250°C; nebulizer gas flow rate: 3 L/min, drying gas flow rate: 15 L/min. Mass spectra were acquired both in positive and in negative full-scan mode in the range of 150 to 1,500 m/z, event time 0.5 s. Product Ion Scan mode (collision energy: -35.0 V for ESI+ and 35.0 V for ESI-, event time: 0.2 s) was applied to obtain more information on the tentatively identified compounds. The experimental data of the UV spectra and mass spectral information for each component were compared to those reported in literature [16-18]; with this approach it was possible to identify them as acylglycosides of apigenin, luteolin, tricetin and chrysoeriol. The tentative identification of the aglycone units was based on the Product Ion Scan results after fragmentation of the protonated $[M+H]^+$ and deprotonated $[M-H]^-$ ions. For the quantification of the acylglycosides the external calibration method based on the liquid chromatography-photodiode array (LC-PDA) profiles of apigenin-7-glucuronide acquired at 336 nm was adopted. A five point's calibration curve was built analyzing in triplicate the pure standard of apigenin-7-glucuronide in the range of 0.5 to 100 µg/mL. The determination coefficient (R^2) was 0.9988.

Animal and experimental design

One hundred and sixty crossbred (Grimaud×Monferrato Grey) weaned rabbits (42 days old) were divided into four groups of forty animals each (twenty males and twenty females), with an initial mean weight of 1,123±160 g. The animals were housed individually in wire cages (41 cm×30 cm×28 cm height), and had free access to clean drinking water. The temperature and photoperiod in the rabbitry were 22°C±2°C and 16 h Lightness:8 h Darkness, respectively. The rabbits were fed a basal diet (AAF0) *ad libitum*, and were tested against three isonitrogenous and isoenergetic diets, supplemented with 400, 800, and 1,200 mg/kg of AAF extract, in which the same quantity of barley meal was replaced. In each experimental group, AAF were mixed in proportion to the premix and then added in diet with remixing. The diets were all pelleted fresh and stored in the dark to prevent auto-oxidation of the lipid sources.

Chemical analyses of the feeds

The analyses were all carried out on three replicates of each feed

sample, according to the recommendations of the European Group on Rabbit Nutrition [19]. The feeds were analyzed for dry matter (# 930.15), ash (# 923.03), crude protein (# 984.13), ether extract (# 2003.05), acid detergent fiber (# 973.18), and acid detergent lignin (# 973.18), according to the recommended AOAC procedures [20,21]. Neutral detergent fiber was determined according to Van Soest et al [22]. Gross energy was measured using an adiabatic bomb calorimeter (C7000, IKA, Staufen, Germany).

Growth performance

During the experiment, the live weight and feed intake were recorded individually on a fortnightly basis. Performance data were recorded over 60 days. Mortality was recorded daily throughout the experimental period. The average daily feed intake, average daily gain, and feed conversion ratio were calculated. Data pertaining to any animal that died were excluded from the calculations of the growth performance parameters.

Slaughter procedures

At 102 days of age, 12 rabbits per group with a weight close to the average of the group (mean weight $3,308 \pm 157$ g) were selected and slaughtered, in a commercial slaughterhouse. The slaughtered rabbits were bled and the skin, genitals, urinary bladder, gastrointestinal tract and distal part of the legs were removed, as recommended by Blasco et al [23]. The carcass was weighed, while the skin and full gastrointestinal tract weights were recorded and expressed as a percentage of slaughter weight (SW). The carcasses (with head, thoracic cage organs, liver, and kidneys) were chilled at 4°C for 24 h in a refrigerated room. The chilled carcass weight (CCW) was recorded, and the dressing out percentage was calculated as the ratio between CCW and SW. Head and liver weight were expressed as percentages of CCW. The head, thymus, trachea, esophagus, heart, lungs, liver and kidney weights were removed from the CCW in order to obtain the reference carcass weight.

Plasma, liver and muscle sampling

Samples of plasma, muscle and liver were collected from 12 animals per group. Blood samples were taken from the saphenous vein using a vacutainer (Venoject, Terumo Europe N.V., Leuven, Belgium) with lithium heparin test tubes. The blood was centrifuged for 15 min at 3,000 rpm, and the resulting plasma samples were stored at -80°C until use. Liver tissues were collected and frozen in liquid nitrogen, and were then stored at -80°C until use. The two loins of each rabbit carcass were used for meat quality and lipid oxidation analyses. The *Longissimus dorsi* (LD) muscle was removed from both left and right sides. The left LD muscle was used to measure pH and color. Samples of the right LD were frozen for a storage period of 30 days at -25°C, and were then analyzed to establish lipid oxidation.

Meat quality parameters

pH: The pH of the meat of the LD muscle was measured (at the

7th lumbar vertebra level) in duplicate 24 h postmortem (pH_{24}) using a Crison portable pH-meter (Crison Instruments, S.A., Alella, Spain), fitted with a spear-type electrode and an automatic temperature compensation probe.

Color: Meat color was measured at room temperature (20°C) on a freshly cut surface of the loin, at the 7th lumbar vertebra level, using a bench colorimeter Chroma Meter CR-400 Konica Minolta Sensing (Minolta Sensing Inc, Osaka, Japan). Color measurements were reported in terms of lightness (L^*), redness (a^*), and yellowness (b^*) in the Commission Internationale de l'Éclairage (CIELAB) color space model [24]. The color values were obtained considering the average of three readings per sample.

Lipid oxidation: Lipid oxidation was determined on meat samples (3 g) after 30 days of frozen storage, by means of a thiobarbituric acid reactive substance (TBARS) assay, as described by Dabbou et al [25]. The samples were analyzed in duplicate, and the absorbance was read at 532 nm using a Helios spectrophotometer (Unicam Limited, Cambridge, UK). The TBARS values were calculated from a standard 1,1,3,3-tetraethoxypropane (TEP) curve (SigmaAldrich, Steinheim, Germany), and the oxidation products were quantified as malondialdehyde (MDA) equivalents (mg MDA/kg of meat).

Tissue oxidative status analysis

Plasma and liver oxidation: The concentration of MDA in the liver and plasma samples was analyzed according to Seljeskog et al [26], with some modifications. The liver samples were homogenized with distilled water 1:10 (w/v) at room temperature in order to prepare them for the MDA extraction. Fifty μL of plasma and the liver samples were then mixed with perchloric acid (0.1125 N, 150 μL) and thiobarbituric acid (TBA; 40 mM, 150 μL), and were vigorously shaken for 10 s and placed in a boiling water bath for 60 min. After cooling in a freezer at -20°C for 20 min, 300 μL of methanol and 100 μL of 20% trichloroacetic acid were added to the suspension and mixed for 10 s. The samples were centrifuged at 10,000 rpm for 6 min, and 10 μL of the supernatant were analyzed by high-performance liquid chromatography (HPLC). A calibration curve was built by analyzing TEP (Sigma Aldrich, Steinheim, Germany) as external standard, dissolved in methanol and diluted at concentrations of 10, 5, 2.5, 1.25, 0.62, and 0.21 μM . The HPLC apparatus consisted of a Dionex P680 pump (Dionex, Sunnyvale, CA, USA), a Dionex RF-2000 fluorimetric detector ($\lambda_{\text{ex}} = 525$, $\lambda_{\text{em}} = 560$), a Dionex thermostated column compartment TCC-100, a Dionex ASI100 autosampler series (Dionex, USA) and a Chromeleon 6 data handling system (Dionex, USA). The analytical column was a Phenomenex Gemini LC-18 column (150 \times 4.6 mm, 5 μm particles) (Phenomenex, Torrance, CA, USA) provided with an Analytical Guard Cartridge System (Phenomenex, USA). The analyses were run isocratically with a mobile phase containing 50 mM KH_2PO_4 -methanol-acetonitrile (72:17:11, v/v), at a flow rate of 0.8 mL/min. The MDA content in the plasma samples was expressed as nmol MDA/mL, and in the liver tissues

as mg MDA/kg of tissue.

Statistical analysis

The statistical analyses were performed using the SPSS software package (version 17 for Windows, SPSS Inc., Chicago, IL, USA). One-way analysis of variance was used to evaluate the effect of the AAF dietary inclusion levels on the carcass characteristics, meat quality traits and tissue oxidative stability. Significance was accepted for $p < 0.05$.

RESULTS AND DISCUSSION

HPLC profile of alfalfa flavonoids extract

Figure 1 reports the HPLC-PDA profile of alfalfa extract and Table 1 represents the spectral information and the relative content of the tentatively identified flavonoids. The total amount of the acylglycosides was 4.4%. A total of 26 components (4.4% of the extract) including feruloylapigenin glycosides, coumaroylapigenin glycosides apigenin and luteolin and tricetin glycosides were found on the basis of their experimental UV and MS data and in accordance with those reported by Stomach et al [16-18]. Stochmal and Oleszek [27] showed that the dominant flavonoids of alfalfa were the flavones: tricetin and apigenin glycosides (around 40% of the total each) while concentration of luteolin and chrysoeriol glycosides did not exceed 10% of the total and these data are similar to our results.

Growth performance

The results of the performance parameter of the rabbits fed the

experimental diets (Table 2) are presented in Table 3. No significant differences in performance traits were found among the treatment groups over the whole experimental period. These results indicate that the supplementation of AAF did not influence the performance of the growing rabbits. These findings are in agreement with those of Dong et al [10], who showed that an aqueous alfalfa extract did not exert any significant effect on the performance of broiler chickens. Deng et al [12] also reported that an aqueous alfalfa extract had no significant effect on the production performance of laying hens.

A similar result was observed by Jiang et al [28] who found that the addition of alfalfa meal did not have any effect on growth performance of Muscovy ducks from the 14th to the 49th day of age. Ouyang et al [29] reported that inclusion of AAF in broiler chickens could improve the growth performance when compared with the control group, especially when the inclusion level was 15 mg/kg diet. In the same context, Chen et al [15] showed that AAF adding in the diet with 300 mg/kg significantly increased feed intake, average daily gain and final weight of Yangzhou geese aged from 28 to 70 days.

Carcass characteristics and meat quality traits

The carcass characteristics and meat quality traits are reported in Table 4. No significant differences were observed among the experimental groups for any of the parameters except for lightness parameter that resulted lower in the control group.

These results are in agreement with those reported for Yangzhou geese by Chen et al [15] who did not find any significant difference for slaughter performance except for the breast muscle

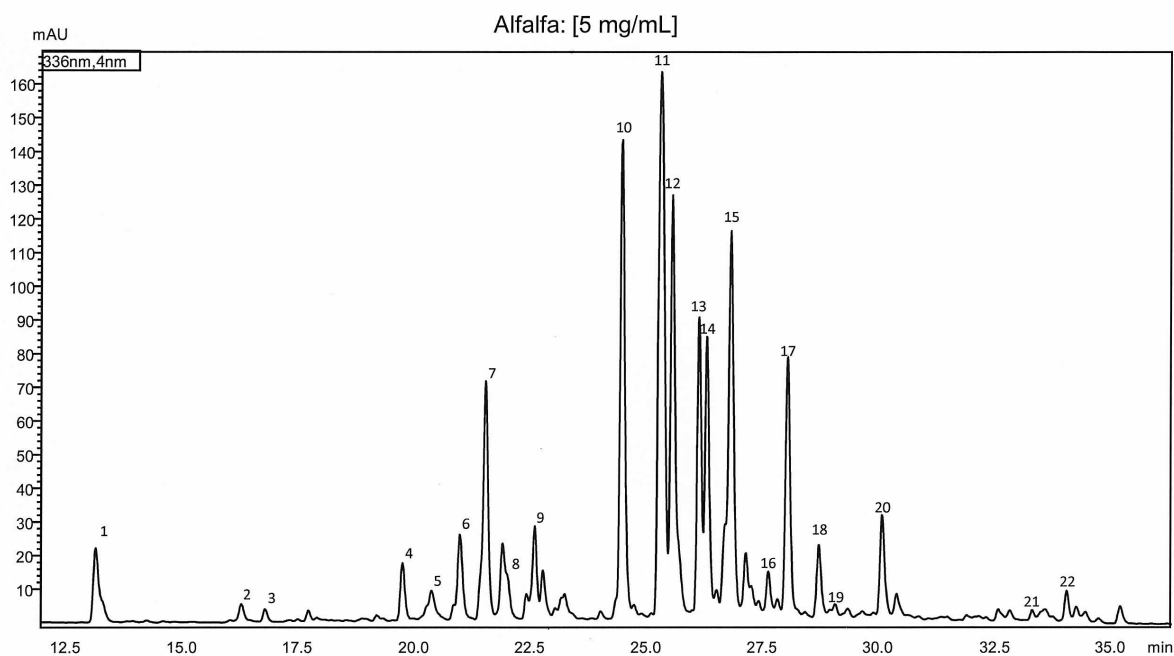


Figure 1. Liquid chromatography coupled with photodiode array profile at 336 nm of alfalfa extract.

Table 1. Spectral data and relative content ($\mu\text{g}/\text{mg}$) of alfalfa flavonoids in water extracts analyzed by HPLC-PDA-MS/MS

Number peak	RT	UV max	MW	Aglycone (MW)	$\mu\text{g}/\text{mg}$	Tentatively identified compounds	Reference
1	13.174	267/321	798	270	1.09	Feruloyl apigenin glycoside/ apigenin glycoside	[16,18]
2	16.309	267/322	622	270	0.14	Apigenin glycoside	[16]
3	16.831	342/266	638	286	0.03	Luteolin glycoside	-
4	19.776	333/266	622	270	0.55	Apigenin glycoside	[16]
5	20.401	265/333	798	270	0.38	Feruloyl apigenin glycoside/ apigenin glycoside	[16,18]
6	21.007	266/348	652	300	0.99	Chrysoeriol glycoside	[17]
7	21.558	348/267	682	330	3.09	Tricin glycoside	[17]
7a	21.558	348/267	622	270	-	Apigenin glycoside	[16]
8	22.021	338/267	462	286	0.34	Luteolin glycoside	[16]
9	22.624	322/270	492	n.i.	1.03	Not identified	-
10	24.498	332/268	974	270	5.49	Feruloylapigenin glycoside	[16]
11	25.338	329/268	944	270	8.19	Coumaroylapigenin glycoside	[18]
12	25.578	338/269	448	286	5.47	Luteolin glycoside	-
12a	25.578	338/269	446	270	-	Apigenin glycoside	[16]
13	26.153	324/269	974	300	3.14	Chrysoeriol glycoside	-
14	26.319	323/271	1004	330	3.03	Tricin glycoside	-
15	26.833	337/270	1012	330	4.96	Tricin glycoside	-
16	27.656	315/271	448	286	0.56	Luteolin glycoside	-
16a	27.656	315/271	944	270	-	Coumaroylapigenin glycoside	[18]
17	28.073	337/269	858	330	3.23	Feruloyltricin glycoside	[17]
17a	28.073	337/269	828	300	-	Feruloylchrysoeriol glycoside	[17]
18	28.751	325/270	828	330	0.90	Coumaroyltricin glycoside	[17]
19	29.103	325/272	828	270	0.08	Apigenin glycoside	-
20	30.113	325/271	798	270	1.27	Feruloyl apigenin glycoside/ apigenin glycoside	[16,18]
21	33.333	325/272	828	330	0.03	Coumaroyltricin glycoside	[17]
22	34.082	330/271	858	330	0.26	Feruloyltricin glycoside	[17]
					44.25		

HPLC-PDA-MS/MS, high-performance liquid chromatography coupled with photodiode array and tandem mass spectrometry; RT, retention time; MW, molecular weight; n.i., not identified.

weight and ratio which were significantly higher than those in the control group.

The ultimate pH in the present study was in the normal range for rabbit meat, as recorded in different studies on rabbit fed diets supplemented with phyto-additives containing carotenoids, tannins and flavonoids [30]. Our result are also in agreement with those reported by Ouyang et al [29] who showed that AAF supplemental diet did not affect the pH value of the breast meat of broiler chickens.

The meat color parameters in the present study were not affected significantly by AAF supplementation except for lightness that resulted lower in the control group.

These results are in agreement with those reported by Ouyang et al [29] who showed that AAF supplemental diet did not affect the meat color of the breast meat of broiler chickens.

The susceptibility of muscle tissue to lipid oxidation can be reduced by means of antioxidants, even though the process depends on several factors. The flavonoids are potent antioxidant agents and protect the cells by scavenging and inhibiting the production and initiation of free radicals, superoxide anions and lipid peroxy radicals [31] or through activation of antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase and

glutathione reductase [32]. Furthermore, the antioxidant properties of lucerne extract have been pointed out by Ben Aziz et al [33]. The effects of alfalfa extract supplementation on the oxidative stability of the LD muscle are shown in Table 4. Dietary AAF supplementation significantly ($p < 0.01$) affected the TBARS values of the frozen meat, with the lowest value (0.24 mg MDA/kg fresh meat) being recorded in the AAF12 group samples (Table 5). The results obtained in our study could be related to the antioxidant activity of AAF compounds [5] which was confirmed by the high percentage (54.42%) of inhibition obtained with the *in vitro* free radical DPPH assay of Alfalfa crude extract. A similar positive influence on rabbit meat lipid oxidation was found in a study carried out on the use as dietary additive of a herbal mixture of ten different herbs and spices rich in flavonoids [34]. These authors suggest that this effect could be related to an enhanced meat antioxidant accumulation.

The literature did not contain information about the effects of dietary supplementation with alfalfa products on lipid oxidation of rabbit meat while few studies are available on pork and poultry meat [8,35-37]. Supplementation of pig diets with alfalfa concentrate at 20 g/kg feed did not result in changes in lipid oxidation of pig meat and cooked ham [35,36]. However, Karwowska

Table 2. Ingredients and chemical composition of the experimental diets

Item	AAF0	AAF4	AAF8	AAF12
Ingredients (g/kg diet)				
Dehydrated alfalfa meal	300	300	300	300
Wheat bran	200	200	200	200
Barley	170	169.6	169.2	168.8
Beet dehydrated pulp	150	150	150	150
Soybean seed meal	115	115	115	115
Molasses	20	20	20	20
Wheat straw	20	20	20	20
Vitamin-mineral premix ¹⁾	15	15	15	15
Soybean oil	5	5	5	5
Bicalcium phosphate	5	5	5	5
Alfalfa flavonoids extract	0	0.4	0.8	1.2
Chemical composition on a dry matter basis				
Dry matter (%)	91.97	91.58	91.75	91.88
Crude ash (%)	7.30	7.75	7.65	7.69
Crude protein (%)	19.24	19.69	19.48	19.50
Ether extract (%)	2.03	2.02	2.03	1.98
Neutral detergent fibre (%)	35.24	35.05	34.69	35.20
Acid detergent fibre (%)	20.62	20.78	20.86	20.90
Acid detergent lignin (%)	3.84	3.81	3.76	3.39
Gross energy (MJ/kg DM)	17.88	17.44	17.93	17.27

AAF, alfalfa flavonoids; DM, dry matter.

¹⁾ per kg of diet: Vit. A 200 UI; α-tocopheryl acetate 16 mg; niacin 72 mg; Vit. B₆ 16 mg; choline 0.48 mg; DL-methionine 600 mg; Ca 500 mg; P 920 mg; K 500 mg; Na 1 g; Mg 60 mg; Mn 1.7 mg; Cu 0.6 mg.

et al [37] suggested that supplementation of turkey diets with 15 and 30 g/kg protein-xanthophyll (PX) concentrate of alfalfa did not result in lower levels of lipid oxidation in breast and thigh muscles. On the other hand, Grell et al [8] reported that 3% addition of PX concentrate of alfalfa to a pig diet was successful in reducing lipid oxidation in the *longissimus* muscle.

Plasma and liver oxidation

MDA is one of the metabolic products of lipid peroxidation that

Table 3. Effect of increasing level of alfalfa flavonoids extract (AAF) on growth performance of growing rabbits (n = 40/group)

	AAF0	AAF4	AAF8	AAF 12	SEM	p-value
FBW (g)	3,448	3,415	3,504	3,444	24.54	0.65
ADFI (g)	136.0	131.1	135.5	128.2	2.12	0.51
ADG (g)	40.1	39.5	41.0	40.0	0.35	0.53
FCR (g/g)	3.4	3.3	3.3	3.2	0.04	0.24

SEM, standard error of the mean; FBW, final body weight; ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio.

is generated by the reaction of lipid oxidation induced by oxygen free radicals in tissues. The MDA levels in the rabbit plasma and liver are reported in Table 5. No significant differences were observed among the groups. Ouyang et al [29] reported that inclusion of AAF decreased the MDA level in the serum in a dose-dependent manner with lower level when AAF was supplemented with 15 mg per kg of diet. In the same context, Jiang et al [28] reported that a dose-dependent increase in the plasma total anti-oxidative activity of broilers by dietary levels of 10 to 80 mg/kg of soy flavonoids. It has been shown that some flavonoids are associated with antioxidant enzyme activity [38], and some of these phyto-molecules contained in alfalfa extract (such as alfalfa isoflavones) could be responsible for this activity.

Table 5. Effect of increasing level of Alfalfa flavonoids extract (AAF) on oxidative status of the muscle, plasma and liver of growing rabbits (n = 12)

	AAF0	AAF4	AAF8	AAF12	SEM	p-value
Muscle (mg MDA/kg of meat)	0.72 ^a	0.35 ^b	0.29 ^{bc}	0.24 ^c	0.03	0.00
Plasma (nmol MDA/mL)	0.18	0.16	0.21	0.19	0.01	0.35
Liver (mg MDA/kg of tissue)	3.09	3.28	2.86	2.85	0.11	0.62

SEM, standard error of the mean; MDA, malondialdehyde.

^{a-c} Different letters within the same row indicate significant differences (p < 0.05).

Table 4. Effect of increasing level of alfalfa flavonoids extract (AAF) on carcass characteristics and *Longissimus dorsi* muscle traits of growing rabbits (n = 12)

	AAF0	AAF4	AAF8	AAF12	SEM	p-value
Slaughter weight (SW, g)	3,383	3,272	3,322	3,259	22.62	0.20
Skin (% SW)	17.9	18.1	17.5	16.1	0.14	0.58
Full gastrointestinal tract (% SW)	14.8	15.5	15.9	16.1	0.19	0.07
CCW (g)	2,092	2,025	2,045	2,002	18.52	0.37
Dressing out (%)	61.8	61.9	61.6	61.4	0.27	0.92
Head (% CCW)	7.26	6.99	7.30	7.52	0.11	0.45
Liver (% CCW)	4.21	4.31	4.28	4.23	0.11	0.17
RCW (g)	1,802	1,747	1,745	1,715	17.73	0.37
pH ₂₄	5.78	5.74	5.75	5.73	0.02	0.73
L* ¹⁾	55.5 ^b	58.2 ^a	57.3 ^{ab}	57.9 ^a	0.35	0.02
a* ¹⁾	-0.08	-1.29	-0.43	-0.79	0.18	0.10
b* ¹⁾	6.1	6.2	6.6	6.4	0.17	0.67

SEM, standard error of the mean; SW, slaughter weight; CCW, chilled carcass weight; RCW, reference carcass weight; pH₂₄, pH at 24 hours post slaughter.

¹⁾ L*, lightness; a*, redness; b*, yellowness.

^{a-b} Different letters within the same row indicate significant differences (p < 0.05).

CONCLUSION

In short, in the present trial, the consumption of different levels of AAF by growing rabbits has not affected the growth performance parameters or the carcass characteristics of growing rabbits. On the other hand, AAF supplementation has been shown to improve muscle oxidation stability through a lowering of the TBARS values in a dose-related manner.

IMPLICATIONS

Herbal feed additives have attracted increasing interest as alternative dietary supplementation in animal production because they have attained more acceptability among consumers as natural feed additives. The supplementation of AAF in rabbit diets improved muscle oxidation stability of the frozen meat in a dose-related manner without affecting growth performance. The current paper demonstrated that alfalfa extract is a suitable herbal additive for rabbit feeds with positive impact on qualitative characteristics of rabbit meat.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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REFERENCES

- Lee MT, Lin WC, Yu B, Lee TT. Antioxidant capacity of phytochemicals and their potential effects on oxidative status in animals- A review. Asian-Australas J Anim Sci 2017;30:299-308.
- Procházková D, Boušová L, Wilhelmová N. Antioxidant and prooxidant properties of flavonoids. Fitoterapia 2011;82:513-23.
- Liang J, Yang Z, Cao X, Wu B, Wu S. Preparative isolation of novel antioxidant flavonoids of alfalfa by stop-and-go counter-current chromatography and following on-line liquid chromatography desalination. J Chromatogr A 2011;1218:6191-9.
- Rafińska K, Pomastowski P, Wrona O, Górecki R, Buszewski B. *Medicago sativa* as a source of secondary metabolites for agriculture and pharmaceutical industry. Phytochem Lett 2017;20:520-39.
- Karimi E, Oskoueian E, Oskoueian A, et al. Insight into the functional and medicinal properties of *Medicago sativa* (alfalfa) leaves extract. J Med Plants Res 2013;7:290-7.
- Goławska S, Łukasik I, Kapusta I, Janda B. Analysis of flavonoids content in alfalfa. Ecol Chem Eng A. 2010;17:261-7.
- Hwang J, Hodis HN, Sevanian A. Soy and alfalfa phytoestrogen extracts become potent low-density lipoprotein antioxidants in the presence of acerola cherry extract. J Sci Food Agric 2001;49:308-14.
- Grela ER, Semeniuk V, Florek M. Effects of protein-xanthophyll (PX) concentrate of alfalfa additive to crude protein-reduced diets on nitrogen excretion, growth performance and meat quality of pigs. J Central Eur Agric 2009;9:669-76.
- KocaoğluGüçlü B, İşcan KM, Uyanik F, Eren M, Can AğcaA. Effect of alfalfa meal in diets of laying quails on performance, egg quality and some serum parameters. Arch Anim Nutr 2004;58:255-63.
- Dong XF, Gao WW, Tong JM, et al. Effect of polysavone (alfalfa extract) on abdominal fat deposition and immunity in broiler chickens. Poult Sci 2007;86:1955-9.
- Dong XF, Gao WW, Su JL, Tong JM, Zhang Q. Effects of dietary polysavone (Alfalfa extract) and chlortetracycline supplementation on antioxidation and meat quality in broiler chickens. Br Poult Sci 2011; 52:302-9.
- Deng W, Dong XF, Tong JM, Xie TH, Zhang Q. Effects of an aqueous alfalfa extract on production performance, egg quality and lipid metabolism of laying hens. J Anim Physiol Anim Nutr 2012;96:85-94.
- Xiong XW, Zhou PF, Ding JH. Effect of alfalfa meal and alfalfa flavonoids excrement on growth performance and nutrient utilization of Chongren chicken female. Jiangxi J Anim Husband Vet Med 2012;3:30-33.
- Zhu JM, Li N, Zhang YJ. Advances in alfalfa flavonoids. Acta Pratacult Sin 2009;26:156-62.
- Chen Y, Gong X, Li G, et al. Effects of dietary alfalfa flavonoids extraction on growth performance, organ development and blood biochemical indexes of Yangzhou geese aged from 28 to 70 days. Anim Sci 2016;2:318-22.
- Stochmal A, Piacente S, Pizza C, et al. Alfalfa (*Medicago sativa* L.) Flavonoids. 1. Apigenin and Luteolin Glycosides from Aerial Parts. J Agric Food Chem 2001;49:753-8.
- Stochmal A, Simonet AM, Macias FA, Oleszek W. Alfalfa (*Medicago sativa* L.) flavonoids. 2. Tricin and chrysoeriol glycosides from aerial parts. J Agric Food Chem 2001;49:5310-4.
- Stochmal A, Simonet AM, Macias FA, et al. Acylated apigenin glycosides from alfalfa (*Medicago sativa* L.) var. Artal. Phytochemistry 2001;8: 1223-6.
- European Group on Rabbit Nutrition. Technical note: attempts to harmonize chemical analyses of feeds and faeces, for rabbit feed evaluation. World Rabbit Sci 2001;9: 57-64.
- AOAC. Official methods of analysis, 17th edn, Association of Official Analytical Chemists, Gaithersburg, MD, USA: AOAC International; 2000.
- AOAC. Official methods of analysis, 17th edn, 2nd revision, Association of Official Analytical Chemists. Gaithersburg, MD, USA: AOAC International; 2003.

22. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fibre, neutral detergent fibre and non starch polysaccharides in relation to animal nutrition. *J Dairy Res* 1991;74: 3583-97.
23. Blasco A, Ouhayoun J, Masoero G. Harmonization of criteria and terminology in rabbit meat research. *World Rabbit Sci* 1993;1:3-10.
24. Commission Internationale de l'Éclairage. Recommendations on uniform colour spaces-colour difference equations, psychometric colour terms (Supplement no. 2 to CIE publication No. 15). Paris, France: Commission Internationale de l'Éclairage; 1976.
25. Dabbou S, Gasco L, Gai F, et al. Dried artichoke bracts in rabbits nutrition: effects on the carcass characteristics, meat quality and fatty-acid composition. *Animal* 2014;8:1547-53.
26. Seljeskog E, Hervig T, Mansoor MA. A novel HPLC method for the measurement of thiobarbituric acid reactive substances (TBARS). A comparison with a commercially available kit. *Clin Biochem* 2006; 39:947-54.
27. Stochmal A, Oleszek W. Seasonal and structural changes of flavones in alfalfa (*Medicago sativa*) aerial parts. *J Food Agric Environ* 2007;5: 170-4.
28. Jiang ZY, Jiang SQ, Lin YC, et al. Effects of soybean isoflavone on growth performance, meat quality, and antioxidation in male broilers. *Poult Sci* 2007;86:1356-62.
29. Ouyang K, Xu M, Jiang Y, Wang W. Effects of alfalfa flavonoids on broiler performance, meat quality and gene expression. *Canad J Anim Sci* 2016;96:332-41.
30. Kovitvadhi A, Gasco L, Ferrocino I, et al. Effect of purple loosestrife (*Lythrum salicaria*) diet supplementation in rabbit nutrition on performance, digestibility, health and meat quality. *Animal* 2016;10:10-8.
31. Kumar S, Pandey AK. Chemistry and biological activities of flavonoids: an overview. *Sci World J* 2013;Article ID 162750.
32. Frankič T, Voljč M, Salobir J, Rezar V. Use of herbs and spices and their extracts in animal nutrition. *Acta Agric Slov* 2009;94: 95-102.
33. Ben Aziz A, Grossman S, Budowski P, Ascarelli I, Bondi A. Antioxidant properties of lucerne extracts. *J Sci Food Agric* 1968;19: 605-8.
34. Mattioli S, Dal Bosco A, Szendrő Zs, Cullere et al. The effect of dietary Digestarom® herbal supplementation on rabbit meat fatty acid profile, lipid oxidation and antioxidant content. *Meat Sci* 2016; 121:238-42.
35. Karwowska M, Dolatowski ZJ, Grela E. The effect of dietary supplementation with extracted alfalfa meal on oxidation stability of cooked ham. *Pol J Food Nutr* 2007;57: 271-4.
36. Karwowska M, Dolatowski ZJ, Grela E. Influence of dietary supplementation with extracted alfalfa meal on meat quality. In: Proceedings of 54th International Congress of Meat Science and Technology; 2008; Cape Town, South Africa.
37. Karwowska M, Stadnik J, Dolatowski ZJ, Grela ER. Effect of protein xanthophylls (PX) concentrate of alfalfa supplementation on physico-chemical properties of turkey breast and thigh muscles during ageing. *Meat Sci* 2010;86:86-90.
38. Ungar Y, Osundahunsi OE, Shimoni E. Thermal stability of genistein and daidzin and its effect on their antioxidant activity. *J Agric Food Chem* 2003;51:4394-9.