Slov Vet Res 2014; 51 (2): 89 - 100
UDC 636.3.087.7:665.345.4:577.161.3:612.12

Original Scientific Article

EFFECT OF DIETARY EXTRUDED LINSEED, VERBASCOSIDE AND VITAMIN E SUPPLEMENTS ON SELECTED SERUM BIOCHEMICAL PARAMETERS AND PLASMA OXIDATIVE STATUS IN LACAUNE EWES

Donato Casamassima[1]*, Maria Nardoia[1], Marisa Palazzo[1], Francesco Vizzarri[2], Carlo Corino[2]

[1]Department of Agricultural, Environmental and Food Sciences, University of Molise, Via De Sanctis, snc - 86100 Campobasso, [2]Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria, 10 - 20133 Milan, Italy

*Corresponding author, E-mail: casamassima.d@unimol.it

Summary: Selected serum biochemical parameters and plasma oxidative status were evaluated in Lacaune ewes on a diet supplemented with extruded linseed, verbascoside and vitamin E. A 98 day-trial was conducted on 44 ewes and started 40 ± 2 days post partum. The animals were divided into four homogeneous groups of eleven animals each; one control group (CON) with a basal diet, and the other three experimental groups supplemented with extruded linseed (L group), extruded linseed-verbascoside (LVB group) and extruded linseed-verbascoside-vitamin E (LVBE group). All animals individually received an isoproteic and isoenergetic diet, consisting of 700g of concentrated feed and meadow hay *ad libitum*. Blood sampling of the ewes was performed three times: at the beginning (0 d), midway (49 d) and end of the trial (98 d). The following parameters were determined: triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, bilirubin, alanine aminotransferase, aspartate aminotransferase, thiobarbituric acid reactive substances (TBARS), reactive oxygen metabolites (ROMs), vitamin A and vitamin E. Dietary integration in the LVB and LVBE groups resulted in an improvement in the blood profile, characterized by a significant (P=0.001) increase in HDL cholesterol and a marked decrease in triglycerides (P=0.001), total cholesterol (P=0.017), LDL cholesterol (P=0.001) and bilirubin (P=0.035), due to the verbascoside supplementation. In the same groups, there was an increase in blood vitamin A (P=0.002) and vitamin E (P=0.001), and a reduction (P=0.001) in ROMs and TBARS, with an improved plasma oxidative status. The dietary vitamin E integration did not produce a significant improvement in the parameters studied, with no statistical differences between LVB and LVBE groups. Animal feed containing extruded flaxseed, might therefore benefit from the addition of a verbascoside supplement, because of the positive effect showed by this molecule on the blood parameters.

Key words: extruded linseed; serum biochemical parameters; verbascoside; vitamin E; Lacaune ewe

Introduction

The integration of feed with extruded linseed is a strategy used in animal feed for milk production in order to improve its acidic profile (1, 2, 3, 4, 5,). Sheep milk fat is characterized by a low concentration of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids and a high content of saturated fatty acids (SFAs), in particular myristic and palmitic acid, with a hypercholesterolemia effect (6, 7). Dietary extruded flaxseed increases the level of MUFA, PUFA and conjugated linoleic acid (CLA) and improves the health benefits of milk and dairy products (5, 8). However, in animals this dietary strategy can lead to an increase in MUFA and PUFA contents in blood, in subcutaneous and intramuscular lipids, thus increasing the susceptibility of these tissues to lipid peroxidation (9, 10).

Received: 2 December 2013
Accepted for publication: 14 April 2014

The use of dietary antioxidants is recommended to limit lipid peroxidation and preserve animal health and product quality (11). Vitamin E is a synthetic antioxidant commonly used in animal nutrition due to its ability to inhibit lipid peroxidation (12) through the inactivation of peroxyl radicals and by capturing radical electrons (13); but its bioefficiency is limited when n-3 PUFA intake is increased (14).

Dietary antioxidant molecules integrated with vitamin E could be more effective for preventing plasma lipid peroxidation (15), as also found by Gladine et al. (10), in dairy cows supplemented with flaxseed oil. This effect was probably because of the hydrophilic properties of plant extracts rich in polyphenols which exhibit affinity for those parts of cells that are complementary to the lipophilic vitamin E (16). Studies on chickens (17) have shown that plant extracts obtained from oregano prevented lipid peroxidation in muscle tissue and may be complementary to vitamin E (17).

Researchers have therefore, focused on characterizing plant extracts, and on isolating and identifying the constituents responsible for antioxidant activities (18, 19). *Lippia citriodora*, a plant species of the Verbenaceae family, is characterized by the presence of different flavonoids and phenolic compounds, including verbascoside (also known as acteoside), and luteolin derivatives (20). Verbascoside is a phenylpropanoid glycoside with anti-inflammatory (21), anti-cancer (22), antioxidant (23) and cardioprotective activities (24). In our previous studies (25, 26, 27, 28), on different animal species, sheep, rabbits and hare, after dietary integration of verbascoside we found a significant improvement in blood parameters, with a decrease in triglycerides, total cholesterol, LDL cholesterol, bilirubin, ROMs and TBARS and an increase in HDL cholesterol, vitamin A and vitamin E. Corino et al. (29) also found a positive effect on plasma oxidation and content of IgA by feeding weaned pigs an extract of *Lippia Citriodora* rich in verbascoside.

The aim of this study was to assess the effects of dietary antioxidant substances, verbascoside and vitamin E, on serum biochemical parameters and plasma oxidative status in milking Lacaune ewes, fed a diet containing extruded flaxseed.

Material and methods

Diet and animals

The trial lasted 98 days and was conducted on 44 Lacaune ewes. At the beginning of the test (40 ± 2 days post partum) the animals were divided into four groups of 11 ewes each, homogeneous by age (4-6 years), body weight (54.06 ± 2.85 kg), parity (III-V) and body condition score (BCS, 2.32 ± 0.11). One group was the control (CON), without the addition of extruded linseed, or feed supplements; while three experimental groups received a dietary supplementation in the feed, of only extruded linseed (L group); extruded linseed and verbascoside (LVB group); and extruded linseed, verbascoside and vitamin E (LVBE group). All ewes were reared in single boxes (size 1.5 x 3.0 m) and all experimental procedures involving animals were in accordance with European Community guidelines and approved by the Italian Ministry of Health.

Each animal received a daily isoproteic and isoenergetic diet as follows:

-CON group: 700 g of basal concentrated pellets, without extruded linseed, and meadow hay *ad libitum*;

-L group: 700 g of concentrated pellets, containing extruded flaxseed 200 g/kg feed, and meadow hay *ad libitum*;

-LVB group: 700 g of concentrated pellets, our formulation, containing extruded flaxseed 200 g/kg feed plus verbascoside 2.86 g/kg feed, and meadow hay *ad libitum*;

-LVBE group: the same dietary treatment as LVB group plus vitamin E 14.29 g/kg feed.

The feed ratio was commensurate with the physiological and productive requirements of milking ewes, according to Nutrient Requirements of Sheep (NRC, 30).

The chemical composition of the feed and meadow hay is reported in Table 1.

The antioxidant supplement contained a water-soluble extract of Verbenaceae (*Lippia* spp.) leaves, prepared on an industrial scale by a standardized procedure which includes ultrasonic extraction with 60% aqueous ethyl alcohol (EtOH) followed by spray drying with maltodextrins as an excipient.

The phenylpropanoid glycoside and benzoic acid content of the feed supplement are reported in Table 2, according to a certificate of analysis provided by the manufacturer. The quantitative analysis of the phenolic compounds was performed by HPLC-UV-DAD (Rastrelli, personal communication) according to Piccinelli et al. (31). To avoid oxidation in the feed, the supplement is microencapsulated within a protective matrix of hydrogenated vegetable lipids using spray-cooling technology (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy).

The dietary vitamin microencapsulated supplement contained 20% of D, L-α-tocopherol acetate, covered with a protective film consisting of a mixture of vegetal fatty acids, and stabilized with a natural antioxidant provided by IZA company (Forlì, Italy).

Blood sampling and analyses

The ewes' blood was blood sampled three times: at the beginning (0 d), midway (49 d) and at the end of the trial (98 d). Blood samples were taken with a vacutainer (Venosafe, Terumo Europe N.V., Leuven, Belgium) from the external jugular vein, on fasted animals for at least 10 hours, with the use of two tubes: the first, with a gel separator, for the production of serum and the second, with lithium heparin for the plasma production. The blood was centrifuged for 15 min at 3000 rpm and the serum immediately tested using an automatic clinical chemistry analyzer, model ARCO (Biotecnica Instruments S.p.A., Rome, Italy) for the following parameters: triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Thiobarbituric acid reactive substance (TBARS) levels of reactive oxygen metabolites (ROMs) and the concentrations of vitamins A and E were determined in the plasma. TBARS was determined in the plasma according to Esterbauer and Zollner (32). Briefly, a standard curve was generated using 1,1,3,3-tetramethoxypropane (Sigma Aldrich, St. Louis, USA). Trichloroacetic acid 10% (v/v) was added to the plasma samples, promoting the precipitation of proteins. The resulting mixture was incubated for 15 min on ice. After centrifugation at 2200 rpm at 4 °C for 15 min, 0.67% thiobarbituric acid was added to the supernatant. The mixture was incubated in a water bath at 90 °C for 10 min, after which the absorbance was read at 532 nm in a spectrophotometer. The results were expressed as µmol of thiobarbituric acid per litre of plasma.

The concentration of ROMs in plasma was determined by a spectrophotometer and a colorimetric method, as proposed by Diacron (Diacron International srl, Grosseto, Italy), using a commercial kit at a wavelength of 505 nm (33). The results were expressed in Carr units (1 Carr unit equals 0.024 mmol/l of H_2O_2).

Vitamins A and E were extracted from the plasma samples with chloroform (34) and analyzed on an HPLC system (Kontron Instruments, Milan, Italy) consisting of an autosampler (HPLC Autosampler 360, Kontron Instruments, Milan, Italy) with a 20 ml loop, a high pressure mixing pump and a 5 µm, 250 x 4.60 mm C18 column (Phenomenex, Torrance, CA, USA). The mobile phase was 100% methanol at a flow rate of 1.0 ml/min. A fluorimeter detector (SFM) and computer with Kroma System 2000 software were used. The vitamin A and E concentrations were determined using an internal standard and the elution time of pure standards.

Statistical analysis

After assessing the frequency distribution, all variables were subjected to analysis of variance using the GLM procedure of the statistical package SPSS (35). Analysis included between-subjects main effect (D) of dietary supplementation (CON, L, LVB and LVBE), within-subjects main effect of sampling time (T) and interaction of dietary supplementation x sampling time (D x T). An individual ewe was the experimental unit. The differences between means were considered significant for $P<0.05$ using the Sheffè test.

Results

Selected serum biochemical parameters

The dietary antioxidant treatment carried out in the LVBE and LVB groups, significantly affected ($P<0.01$) the lipid profile parameters, such as triglycerides, total cholesterol, HDL cholesterol and LDL cholesterol (Table 3).

Table 1: Chemical composition and fatty acid profile of diets

		Concentrates[1]		Hay
		TypeA[2] (CON group)	**Type B[3]** (L, LVB, LVBE groups)	
Chemical composition	**%**			
Dry matter		923.0	929.9	92.20
Crude protein		178.0	180.2	7.80
Ether extract		33.6	125.1	1.80
Ash		70.0	81.9	7.50
Neutral detergent fiber (NDF)		169.9	249.1	57.60
Acid detergent fiber (ADF)		47.8	74.9	39.50
Lignin		10.9	22.7	5.50
Calcium		12.6	13.8	1.10
Phosphorus		3.3	4.0	0.25
Fatty acid	**g100·g^{-1} FA**			
C 16:0		15.21	9.60	19.00
C 18:0		1.99	4.14	3.20
C 18:1 n-9		22.98	19.20	6.55
C 18:2 n-6		54.15	21.92	26.95
C 18:3 n-3		3.52	43.95	36.90
Others fatty acid		2.15	1.19	7.40
Metabolizable energy	**Mcal·kg^{-1}**	3260	3300	1355

[1]**Ingredients of concentrate pellets**: barley, soybean flour, corn flour, extruded linseed (for experimental groups only) wheat bran, wheat flour, molasses, di-calcium phosphate, calcium carbonate, sodium chloride, sodium bicarbonate, magnesium oxide. Minerals and vitamins supplement /kg of concentrate: iron ($FeSO_4$) 100 mg; iodine ($Ca(IO_3)_2$) 5.00 mg; cobalt ($CaSO_4$) 3.00 mg; zinc (ZnO) 100 mg; selenium (Na_2SeO_3) 0.30 mg. Vitamins: A 40,000 U.I; D3 4,000 U.I; E acetate 40 mg; B_1 3.0 mg; B_2 2.0 mg; B_6 0.40 mg; B_{12} 0.010 mg.

[2]**Type A**: - control concentrate without extruded linseed and dietary supplements (*CON group*)
[3]**Type B**: - experimental concentrate with 200 g of extruded linseed·kg^{-1} of concentrate (*L group*)
- experimental concentrate with 200 g of extruded linseed + 2.86 g of verbascoside supplement·kg^{-1} of concentrate *(LVB group)*
- concentrate with 200 g of extruded linseed + 2.86 g of verbascoside supplement + 14.29 g of microencapsulated vitamin E supplement (D, L-α tocoferil acetate)·kg^{-1} of concentrate (*LVBE group)*

Table 2: Phenylpropanoid glycosides and benzoic acid content of feed supplement

Components	**g·kg^{-1} of supplement**
Gallic acid	1.755 ± 0.07
3.4-dihydroxybenzoic acid	0.450 ± 0.04
Methyl gallate	1.955 ± 0.09
Isoverbascoside	0.455 ± 0.04
Verbascoside	4.470 ± 0.08

Table 3: Serum biochemical parameters in Lacaune ewes

Parameters		Groups†				SEM§	P-value‡		
		CON	L	LVB	LVBE		D	T	D x T
Ewes	**n.**	11	11	11	11				
Triglycerides	**mg·dl^{-1}**								
0 d		19.87^{a}	20.14^{a}	21.30^{a}	22.90^{a}	0.569			
49 d		22.75	23.66	19.58	21.63	0.619			
98 d		25.86^{1b}	26.76^{1b}	18.20^{2b}	19.40^{2b}	0.709	0.001	0.035	0.001
Total cholesterol	**mg·dl^{-1}**								
0 d		72.85	73.76	74.62^{a}	73.24^{a}	0.479			
49 d		74.02	74.93	72.23	71.19	0.461			
98 d		73.95^{1}	74.86^{1}	70.51^{2b}	69.89^{2b}	0.579	0.017	0.021	0.047
HDL cholesterol	**mg·dl^{-1}**								
0 d		26.76	26.40	27.42^{a}	26.93^{a}	0.368			
49 d		26.93^{1}	26.75^{1}	29.35^{2}	29.60^{2}	0.379			
98 d		26.12^{1}	26.31^{1}	33.69^{2b}	32.90^{2b}	0.680	0.001	0.001	0.001
LDL cholesterol	**mg·dl^{-1}**								
0 d		41.71	42.62	39.14^{a}	38.36^{a}	0.613			
49 d		43.97^{1}	39.14^{1}	36.32^{2}	36.34^{2}	0.895			
98 d		45.03^{1}	45.58^{1}	34.89^{2b}	34.72^{2b}	0.944	0.001	0.017	0.116
ALT	**UI·l^{-1}**								
0 d		36.19	35.65	38.04^{a}	38.70^{a}	0.390			
49 d		38.88	37.98	37.14	38.09	1.150			
98 d		37.90	37.00	34.03^{b}	35.82^{b}	0.480	0.484	0.015	0.308
AST	**UI·l^{-1}**								
0 d		74.50	74.05	74.92	72.80	1.300			
49 d		73.98	73.53	72.01	71.78	0.890			
98 d		74.71	74.17	72.39	72.24	2.550	0.140	0.373	0.839
Bilirubin	**mg·dl^{-1}**								
0 d		0.534	0.536	0.532^{a}	0.540^{a}	0.018			
49 d		0.547	0.543	0.517	0.522	0.011			
98 d		0.548^{1}	0.553^{1}	0.505^{2b}	0.488^{2b}	0.025	0.035	0.029	0.011

Table 4: Plasma oxidative status marker in Lacaune ewes

Item		Groups†				SEM§	P-value‡		
		CON	L	LVB	LVBE		D	T	D x T
Ewes	**n.**	11	11	11	11				
ROMs	**U·Carr^{-1}**								
0 d		158.69^{a}	163.28^{a}	160.17^{a}	161.65^{a}	2.74			
49 d		261.92^{1b}	266.44^{1b}	133.58^{2b}	138.28^{2b}	10.41			
98 d		289.33^{1c}	298.42^{1c}	119.23^{2c}	116.62^{2c}	15.73	0.001	0.001	0.001
TBARS	**µmol·l^{-1}**								
0 d		0.212^{a}	0.202^{a}	0.194^{a}	0.208^{a}	0.029			
49 d		0.296^{1b}	0.323^{1b}	0.176^{2}	0.182^{2}	0.022			
98 d		0.481^{1c}	0.535^{1c}	0.138^{2b}	0.140^{2b}	0.031	0.001	0.003	0.001
Vitamin E	**µmol·l^{-1}**								
0 d		0.165	0.164	0.163^{a}	0.160^{a}	0.008			
49 d		0.157^{1}	0.166^{1}	0.334^{2b}	0.311^{2b}	0.020			
98 d		0.162^{1}	0.168^{1}	0.541^{2c}	0.478^{2c}	0.042	0.001	0.001	0.001
Vitamin A	**µg·ml^{-1}**								
0 d		0.145	0.148	0.143^{a}	0.150^{a}	0.008			
49 d		0.146^{1}	0.153^{1}	0.185^{2b}	0.201^{2b}	0.010			
98 d		0.147^{1}	0.152^{1}	0.221^{2b}	0.264^{2b}	0.012	0.002	0.001	0.006

† CON- control diet; L- 200g of extruded linseed·kg^{-1} of concentrate; LVB-200 g of extruded linseed + 2.86 g of verbascoside supplement based·kg^{-1} of concentrate; LVBE- 200 g of extruded linseed + 2.86 g of verbascoside supplement + 14.29 g of micro-encapsulated vitamin E·kg^{-1} of concentrate; § SEM= Standard error of mean; ‡ D= fixed effect of dietary supplementation; T=fixed effect of time; D x T= interaction dietary supplementation x time; ^{1,2} within a row, means without a common superscript differ (P<0.05); $^{a, b}$ within a column, means without a common superscript differ (P<0.05).

Triglycerides, at the end of the trial (98d), significantly decreased (P<0.01) in LVB and LVBE groups by 29.6% and 25.0% compared to the CON group and by 32.0% and 27.5% compared to the L group, respectively. No statistical difference was found between the LVB and LVBE groups. In addition, from the first to the third sampling, an effect of dietary treatment was observed with a significant triglycerides reduction (P<0.05) in the LVB and LVBE groups; while, a significant increase in values (P<0.05) was reported in the CON and L groups in the same period.

Total cholesterol was influenced by the experimental treatment (P<0.05) at the last sampling, with a decrease of 4.6% and 5.5% in LVB and LVBE groups compared to the CON group, and by 5.8% and 6.6% compared to the L group, respectively. No statistical difference between the LVB and LVBE groups was found. The dietary time effect on the serum concentration of total cholesterol was significant, from the first to the third sampling, which showed a significant decrease (P<0.05) in the LVB and LVBE groups. In the same period of time, the CON and the L group did not present significant variations.

At the end of the test, the dietary treatment led, to a significant increase in HDL cholesterol (P<0.01) in the LVB and LVBE groups, by 29.0% and 26.0% compared to the CON group, and by 28.0% and 25.0% compared to the L group, respectively. No statistical difference between the LVB and LVBE groups was found. From the first to the third sampling the time of treatment highlighted a significant increase (P<0.01) of HDL cholesterol values in the LVB and LVBE groups In the same period of time, the CON and the L group did not show any significant variations.

LDL cholesterol was statistically affected (P<0.01) by dietary treatment, at the end of the test (98d), with a reduction of values by 22.5% and 22.9% in the LVB and LVBE groups compared to the CON group, and by 23.5% and 23.8% compared to the L group, respectively. No difference between the LVB and LVBE groups was found. From the beginning to the end of the trial, the dietary time effect determined a significant reduction (P<0.05) in LDL cholesterol in the LVB and LVBE groups, while values in the CON group and L group remained essentially unchanged.

The AST and ALT values (Table 3) were not affected by dietary treatment; however there was a significant decrease in ALT values (P<0.05) over time in the LVB and LVBE groups. There were no significant changes in the CON and L group over the same period of time. Bilirubin concentration (Table 3) was not significantly affected (P<0.05) by dietary treatment, at the end of the test, with a decrease in values of 7.8% and 10.9% in the LVB and LVBE groups compared to the CON group, and 8.7% and 11.7% compared to the L group, respectively. No statistical difference by dietary treatment between the LVB and LVBE groups was observed. The treatment time effect showed a significant reduction in values (P<0.05) in the LVB and LVBE groups, while values in the CON and L group remained almost unchanged.

All the parameters in Table 3, except LDL cholesterol, ALT and AST, showed a significant interaction of experimental treatment in relation to the time of administration, highlighting significant modifications of values over the time of administration.

Plasma oxidative status markers

At the end of the test (98d), ROMs values (Table 4) showed a significant decrease (P<0.01) of 58.8% and 59.7% in the LVB and LVBE groups compared to the CON group, and 60.0% and 60.9% compared to the L group, respectively. Statistical differences were determined as early as the midway-trial sampling (49 d) and continued until the end of the trial. No statistical difference between the LVB and LVBE groups was found. The dietary time effect was also significant (P<0.01) throughout the trial, with a reduction of ROMs values in the LVB and LVBE groups; while in the same time period ROMs increased by 82.3% in the CON group and 82.7% in the L group.

At the end of the trial TBARS values (Table 4) were also significantly (P<0.01) lower by 71.3% and 70.9% in LVB and LVBE groups compared to the CON group, and by 74.2% and 73.8% compared to the L group, respectively. This difference is highlighted, from the second sampling, with a decrease in values in the LVB and LVBE groups and an increase in the CON and L groups. TBARS also did not register any significant difference between the LVB and LVBE groups. The duration of the dietary treatment, from the first to the third sampling, in the LVB and LVBE groups, showed a statistical decrease (P<0.01) of TBARS values; while in the CON and L groups in the same period, there was an increase of 126.9% values and 164.8%, respectively.

The administration of feed supplements in the LVB and LVBE groups produced a significant increase (P<0.01) in vitamin E concentration (Table 4) from sampling day 49 until 98 d where the increase was 233.9 % and 195.1%, compared to the CON group, and 222% and 184.5%, compared to the L group, respectively. No statistical difference in vitamin E concentration was recorded between the LVB and LVBE groups. The increase in vitamin E was markedly high (P<0.01) over time, and from the first to the third sampling increased by 231.9% and 198.8% in the LVB and LVBE groups; while over the same period in the CON and L groups, the vitamin E concentration remained unchanged.

At the end of the test, the Vitamin A concentration (Table 4) increased significantly (P<0.01) in the LVB and LVBE groups by 50.3% and 79.6%, compared to the CON group, and by 45.4% and 73.7%, compared to the L group, respectively. The vitamin A concentration increased in the second sampling, in both LVB and LVBE groups, while the CON and L groups values remained almost unchanged. No statistical difference between the LVB and LVBE groups was found. There was a significant (P<0.01) time effect in the LVB and LVBE groups from the first to the third sampling, while, at the same time the CON and L values remained unchanged.

Plasma oxidative status markers showed a significant interaction (P<0.01) of dietary treatment for the duration of trial was observed.

Discussion

Selected serum biochemical parameters

Ewes in the LVB and LVBE groups that were fed dietary antioxidant supplements were found to have a significant improvement in the lipid blood profile parameters, with a decrease in triglycerides, total cholesterol and LDL cholesterol and a marked increase in HDL cholesterol. This increase in HDL cholesterol was due to the exclusive action of verbascoside, in fact there were no additional effects with vitamin E supplementation in the LVBE group.

The L group on the other hand presented similar values to the CON group, in agreement with the findings reported by Bouattour (36) in Lacaune ewes, and Gobert et al (15) and Petit et al. (8) in Holstein Frisian cows fed with linseed.

It is known that the hepatic formation of cholesterol is partly dependent on the availability of HMG-CoA reductase enzymes and partly on the presence of LDL receptors. Verbascoside acts on the lipid metabolism in similar way to statins (simvastatin and pravastatin), which are used in the treatment of hypercholesterolemia to reduce cholesterol synthesis and to increase the expression of LDL receptors by acting at the level of HMG-CoA, an enzyme involved in cholesterol synthesis in the liver. Inhibition of cholesterol synthesis in the liver results in an up-regulation of liver LDL receptor expression and a concomitant decrease in plasma LDL receptor concentration (37).

Similarly, Shimoda et al. (38) attributed the decrease in blood cholesterol levels in mice, fed an extract of *Cistanche tubulosa*, to the capacity of verbascoside to inhibit the expression of the RNA messenger for enzymes involved in the synthesis of cholesterol, such as HMG-CoA reductase and mevalonate kinase, and to the increase in the transport and metabolism of cholesterol. In addition, Shimoda et al. (38) reported an increase in the expression of apolipoprotein B, the VLDL receptor and lipoprotein lipase, involved in the transport and storage of cholesterol. They also found an increase in lipin1, peroxisome proliferator-activated alpha (PPARα) receptor, cytochrome P450 and other molecules involved in cholesterol metabolism.

Nammi et al. (39) showed that the administration of ethanolic extract of ginger (*Zingiber officinalis*) in high-fat diet rats, significantly reduced blood levels of total cholesterol, LDL cholesterol and triglycerides. They also observed that ginger causes an *up-regulation* of the gene expression, coding for the LDL receptor, and a *down-regulation* of HMG-CoA reductase in target tissues of rats. Other Authors (40) reported that polyphenols activated the PPARα receptor, by modulating the expression of key proteins involved in the HDL metabolism in the liver. In studies on rats fed a leaf extract of *Gmelina arborea* (Verbenaceae family), rich in lignans, flavonoids and phenylpropanoids glycosides, Punitha et al. (41) reported a decrease in blood levels of total cholesterol, LDL cholesterol, triglycerides and an increase in HDL cholesterol, due to a reduced hepatic triglyceride synthesis and/or a reduction in lipolysis.

The lack of the effect of vitamin E on the blood lipid profile in the LVBE group is in agreement with Soliman et al. (42), who reported no significant changes in blood total lipids, in sheep and lambs fed with vitamin E and selenium. Administering α-tocopherol in growing lambs, Njeru et al. (43), found no significant increases in serum cholesterol and triglycerides. Studies conducted by Yang et al. (44) on lactating goats, reported no appreciable changes in plasmatic values of triglycerides and total cholesterol, after injection of 3000 IU D-alpha-tocopherol acetate.

In our findings, dietary treatment did not produce a significant improvement in the plasmatic concentrations of hepatic markers, such as ALT and AST, except for bilirubin, whose values significantly decreased in the LVB and LVBE groups compared to the CON and L groups. The decrease in bilirubin, is likely due to the action of verbascoside, in fact there were no statistical differences from LVB group to LVBE group. Nudda et al. (4), reported no significant variations in bilirubin and AST blood levels in goats fed with extruded flaxseed supplement. Aliyu et al. (45) showed a progressive decrease in blood bilirubin levels in rats, after administration of an aqueous extract of bark of *Boswellia dalzielii*, a plant typical of Nigeria, rich in phenolic compounds. They reported that this extract may have had a protective effect on red blood cells, which are precursors of bilirubin, and the liver. Similar results were also obtained by Onoriose et al. (46) in rats treated with CCL4 and fed with a leaf extract of the same plant.

Plasma oxidative status markers

The significant decrease in the LVB and LVBE groups regarding the plasma oxidative status markers, compared to the CON and L groups, is likely due to the verbascoside supplement because no statistical differences between the LVB and the LVBE groups were found. Verbascoside oxidizes and shrinks without becoming a highly-reactive-radical molecule, it also has a preventive function against reactive oxygen substances (ROS), with a consequent reduction in lipid peroxidation. The verbascoside activity can be attributed both to direct action (the trapping of free radicals by verbascoside due to its antioxidant activity during the propagation of chain oxidative reactions) and a blocking of the initiation phase of oxidation through the inhibition of pro-oxidant enzymes which are responsible for the production of free radicals (26).

By testing MDA, Funes et al. (47), showed how verbascoside can inhibit lipid peroxidation. The verbascoside showed a powerful antioxidant property, stronger than hydroxytyrosol and caffeic acid, and similar to quercetin. The ROS scavenger capacity of phenylpropanoid glycosides depends on the number of phenol-hydroxyl groups. In fact, the higher the number of these groups the greater the antioxidant activity, and the verbascoside antioxidant activity is due to the presence of four phenol-hydroxyl groups (48). Despite being a water-soluble compound, verbascoside can prevent lipid peroxidation, probably due to some molecular interaction with the surface of the lipid membrane, as has been proposed by Funes et al. (47) when testing other hydrophilic antioxidant substances. Liu et al. (49) reported a lower plasma value of TBARS in rabbits fed twice a day with verbascoside (0.8 mg/kg of body weight). In a study on the skeletal muscle of rats subjected to strain, whose diet (for 10 days) was supplemented with verbascoside, Liao et al. (50), observed a decrease in ROM concentrations than untreated animals. In a study on lactating cows, fed with extruded flaxseed, vitamin E and vegetable extracts rich in polyphenols, Gobert et al. (15) found that only vitamin E did not affect the plasma lipid peroxidation, while in association with other herbal extracts vitamin E prevents oxidative damage, as was highlighted in our study by the lower levels of plasma concentration of TBARS in treated groups compared to the control group.

The increased values of ROMs and TBARS in the L group are likely due to the high content of linseed α-linolenic acid and PUFA (51), which is one of the factors that can influence and increase the oxidation of tissues (52).

The increase in plasma vitamins E and A in the LVB and LVBE groups, might be due to verbascoside ability to preserve the endogenous antioxidant system, through a reduction in reactive radical species in plasma (26). The dietary supplement integration of vitamin E did not produce significant improvements in plasma vitamin A and E concentrations. The increase in plasma vitamin E could also be attributed to the ability of verbascoside to enhance the α-tocopheroxyl radical recycling, as reported for green tea polyphenols (53), or to encourage the accumulation of α-tocopherol, following a reduction in oxidative-propagation-phase activation. The increase in vitamin A on the other hand, after verbascoside integration, could be due to an improvement in the β-carotene conversion process in vitamin A, made by β-carotene 15, 15' monooxygenase enzyme (a precursor of retinol), as evidenced by Yang and Tume (54).

In conclusion, the dietary integration of extruded linseed, verbascoside and vitamin E, resulted in an improvement in the blood profile. This profile was characterized by an increase in HDL cholesterol and a significant reduction in triglycerides, total cholesterol, LDL cholesterol and bilirubin, in LVB and LVBE treated groups, due to the verbascoside supplement.

The verbascoside also resulted in improved plasma oxidative status characterized by a significant increase in plasma vitamin A and vitamin E concentrations and a significant reduction in ROMs and TBARS values. The dietary vitamin E integration did not produce a significant improvement in the studied parameters, with no statistical differences between the LVB and LVBE groups.

Animal feed containing extruded flaxseed, might therefore benefit from a verbascoside supplement, because of the positive effect shown by this molecule on biochemical parameters.

References

1. Caroprese M, Albenzio M, Bruno A, Fedele V, Santillo A, Sevi A. Effect of solar radiation and flaxseed supplementation on milk production and fatty acid profile of lactating ewes under high ambient temperature. J Dairy Sci 2011; 94: 3856–67.

2. Gómez-Cortés P, Bach A, Luna P, Juárez M, de la Fuente MA. Effects of extruded linseed supplementation on n-3 fatty acids and conjugated linoleic acid in milk and cheese from ewes. J Dairy Sci 2009; 92: 4122-34.

3. Gonthier C, Mustafa AF, Ouellet DR, Chouinard PY, Berthiaume R, Petit HV. Feeding micronized and extruded flaxseed to dairy cows: effects on blood parameters and milk fatty acid composition. J Dairy Sci 2005; 88: 748–56.

4. Nudda A, Battacone G, Atzori AS, et al. Effect of extruded linseed supplementation on blood metabolic profile and milk performance of Saanen goats. Animal 2013; 7 (9): 1464–71.

5. Zhang RH, Mustafa AF, Zhao X. Effects of flaxseed supplementation to lactating ewes on milk composition, cheese yield, and fatty acid composition of milk and cheese. Small Rumin Res 2006; 63: 233–41.

6. Haenlein GFW. Nutritional value of dairy products of ewe and goat milk. In: Seminar production and utilization of ewe and goat milk: proceedings of IDF Greek National Committee of IDF/CIRVAL. Greece, Crete, 1996; 3: 348.

7. Kennelly JJ. The fatty acid composition of milk fat as influenced by feeding oilseeds. Anim Feed Sci Tech 1996; 60: 137–52.

8. Petit HV. Digestion, milk production, milk composition, and blood composition of dairy cows fed whole flaxseed. J Dairy Sci 2002; 85: 1482–90.

9. Scislowski V, Bauchart D, Gruffat D, Laplaud PM, Durand D. Effects of dietary *n* − 6 or *n* − 3 polyunsaturated fatty acids protected or not against ruminal hydrogenation on plasma lipids and their susceptibility to peroxidation in fattening steers. J Anim Sci 2005; 83: 2162–74.

10. Gladine C, Morand C, Rock E, Bauchart D, Durand D. Plant extracts rich in polyphenols (PERP) are efficient antioxidants to prevent lipoperoxidation in plasma lipids from animals fed *n* − 3 PUFA supplemented diets. Anim Feed Sci Techn 2007; 136 (3/4): 281–96.

11. Wood JD, Enser M. Factors influencing fatty acids in meat and the role of antioxidants in improving meat quality. Br J Nutr 1997; 78: S49–S60.

12. Cuvelier C, Dotreppe O, Istasse L. Chemistry, nutritional sources and analyses of vitamin E. Ann Med Vet 2003; 147: 315–24.

13. Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. FASEB J 1999; 13: 1145–55.

14. Allard J, Kurian R, Aghdassi E, Muggli R, Royall D. Lipid peroxidation during *n* − 3 fatty acid and Vitamin E supplementation in humans. Lipids 1997; 32: 535–41.

15. Gobert M, Martin B, Ferlay A, et al. Plant polyphenols associated with vitamin E can reduce plasma lipoperoxidation in dairy cows given n-3 polyunsaturated fatty acids. J Dairy Sci 2009; 92: 6095–104.

16. Yeum KJ, Russell RM, Krinsky NI, Aldini G. Biomarkers of antioxidant capacity in the hydrophilic and lipophilic compartments of human plasma. Arch Biochem Biophys 2004; 430 (1): 97–103.

17. Papageorgiou G, Botsoglou A, Govaris A, Giannenas I, Iliadis S, Botsoglou E. Effects of dietary oregano oil and α-tocopheryl acetate supplementation on iron-induced lipid oxidation of turkey breast, thigh, liver and heart tissues. J Anim Physiol Anim Nutr 2003; 87: 324–35.

18. Valentão P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML. Antioxidant activity of *Centaurium erythraea* infusion evidenced by its superoxide radical scavenging and xanthine oxidase inhibitory activity. J Agric Food Chem 2001; 49: 3476–9.

19. Soares JR, Dins TCP, Cunha AP, Almeida LM. Antioxidant activity of some extracts of Thymus zygis. Free Radic Res 1997; 26: 469–78.

20. Valentão P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML. Studies on the antioxidant activity of *Lippia citriodora* infusion: scavenging effect on superoxide radical, hydroxyl radical and hypochlorous acid. Bio Pharm Bull 2002; 25: 1324–7.

21. Deepak M, Handa SS. Anti-inflammatory activity and chemical composition of extracts of *Verbena officinalis*. Phytother Res 2000; 14: 463–5.

22. Kunvari M, Paska C, Laszlo M, et al. Biological activity and structure of antitumor 60 compounds from *Plantago media* L. Acta Pharm Hung 1999; 69 (5): 232–9.

23. Daels-Rakotoarison DA, Seidel V, Gressier B, et al. Neurosedative and antioxidant activities of phenylpropanoids from ballota nigra. Arzneimittelforschung 2000; 50(1): 16–23.

24. Pennacchio M, Alexander E, Syha YM, Ghisalberti EL. The effect of verbascoside on cyclic 3',5'-adenosine monophosphate levels in isolated rat heart. Eur J Pharmacol 1996; 305: 169–71.

25. Casamassima D, Palazzo M, Presutti T, Colella G. Productive performances, plasmatic

oxidative status and some blood parameters in suckling lambs supplemented with verbascoside. In: Proceedings of the 18th Animal Science and Production Association Congress. Palermo, 2009: 668.

26. Casamassima D, Palazzo M, Martemucci G, Vizzarri F, Corino C. Effects of verbascoside on plasma oxidative status and blood and milk production parameters during the peripartum period in Lacaune ewes. Small Rumin Res 2012; 105: 1–8.

27. Casamassima D, Palazzo M, Vizzarri F, Cinone M, Corino C. Effect of dietary phenylpropanoid glycoside-based natural extracts on blood parameters and productive performance in intensively-reared young hares. Czech J Anim Sci 2013; 58 (6): 270–8.

28. Palazzo M, Vizzarri F, Cinone M, Corino C, Casamassima D. Assessment of a natural dietary extract, titrated in phenylpropanoid glycosides, on blood parameters and plasma oxidative status in intensively reared Italian hares (*Lepus corsicanus*). Animal 2011; 5 (6): 844–50.

29. Corino C, Rossi R, Musella M, Cannata S, Pastorelli G. Growth performance and oxidative status in piglets supplemented with verbascoside and teupolioside. Ital J Anim Sci 2007; 6: 292–4.

30. National Research Council. Nutrient requirements of sheep. 6th ed. Washington : National Academy Press, 1985.

31. Piccinelli AL, DeSimone F, Passi S, Rastrelli L. Phenolic constituents and antioxidant activity of *Wendita calysina* leaves (Burrito), a folk paraguayan tea. J Agric Food Chem 2004; 52: 5863–8.

32. Esterbauer H, Zollner H. Methods for determination of aldehydic lipid peroxidation products. Free Radic Biol Med 1989; 7: 197–203.

33. Cesarone MR, Belcaro G, Caratelli M. A simple test to monitor oxidative stress. Int J Angiol 1999; 18: 127–30.

34. Zhao B, Tham SY, Lu J, Lai MH, Lee LKH, Moochhala SM. Simultaneous determination of vitamins C, E and β carotene in human plasma by high-performance liquid chromatography with photodiode-array detection. J Pharm Pharmaceut Sci 2004; 7: 200–4.

35. SPSS, 19.0 Package program. User's guide. Chicago : SPSS, 2011.

36. Bouattour MA. Effects of feeding different vegetal fat sources to increase conjugated linoleic acid in milk of small ruminants and interaction with fibrolytic enzymes: thesis dissertation. Barcelona : Universitat Autònoma , 2007.

37. Chen ZY, Jiao R, Ma KY. Cholesterol-lowering nutraceuticals and functional foods. J Agric Food Chem 2008; 56: 8761–73.

38. Shimoda H, Tanaka J, Takahara Y, Takemoto K, Shan SJ, Su MH. The hypocholesterolemic effects of *Cistanche tubulosa* extract, a chinese traditional crude medicine, in mice. Am J Chinese Med 2009; 37: 1125-38.

39. Nammi S, Kim MS, Gavande NS, Li GQ, Roufogalis BD. Regulation of low-density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase expression by *Zingiber officinale* in the liver of high-fat diet-fed rats. Basic Clin Pharmacol Toxicol 2009; 106: 389–95.

40. Norata GD, Pellegatta F, Catapano AL. Peroxisome proliferator actived receptors and cardiovascular disorders. Ital Heart J 2003; 4: 8–18.

41. Punitha D, Thandavamoorthy A, Arumugasamy K, Suresh SN, Danya U, Udhayasankar MR. Anti-hyperlipidemic effect of ethanolic leaf extract of *Gmelina arborea* in streptozotocin induced male wistar albino rats. Int J Life Sci Pharma Res 2012; 2 (3): 46–51.

42. Soliman EB, Abd El-Moty AKI, Kassab AY. Combined effect of vitamin E and selenium on some productive and physiological characteristics of ewes and their lambs during suckling period. Egypt J Sheep Goat Sci 2012; 7(2): 31–42.

43. Njeru CA, McDowell LR, Wilkinson NS, Williams SN. Assessment of vitamin E nutritional status in sheep. J Anim Sci 1994; 72: 3207–12.

44. Yang DY, Chang CJ, Peh HC, Chen MT. Anti-peroxidation effects of vitamin E on low density lipoprotein and milk fat globule membrane of lactating goats: in vivo versus metal ion challenge in vitro. Comp Biochem Phys 2004; 139: 11–20.

45. Aliyu R, Gatsing D, Jaryum KH. The effect of *Boswellia dalzielii* (Buseraceae) aqueous bark extract on rat liver function. Asian J Biochem 2007; 2 (5): 359–63.

46. Onoriose DA, Uwakwe AA, Monago CC, Odeghe OB. Hepatoprotective effect of methanolic leaf extract of *Boswellia dalzielii* hutch on carbon tetrachloride induced hepatotoxicity in wistar rats. Indian J Med Healthcare 2012; 1(3): 2278–334.

47. Funes L, Fernandez AS, Laporta O, et al. Correlation between plasma antioxidant capacity and verbascoside levels in rats after oral administration of lemon verbena extract. Food Chem 2009; 17: 589–98.

48. Pastorelli G, Rossi R, Corino C. Influence of *Lippia citriodora* verbascoside on growth performance, antioxidant status, and serum immunoglobulins content in piglets. Czech J Anim Sci 2012; 57(7): 312–22.

49. Liu MJ, Li JX, Guo HZ, Lee KM, Qin L, Chan KM. The effects of verbascoside on plasma lipid peroxidation level and erythrocyte membrane fluidity during immobilization in rabbits: a time course study. Life Sci 2003; 73: 883–92.

50. Liao F, Zheng RL, Gao JJ, Jia ZJ. Retardation of skeletal muscle fatigue by the two phenylpropanoid glycosides: verbascoside and martynoside from *Pedicularis plicata* Maxim Phytother Res 1999; 13: 621–3.

51. Trebušak T, Levart A, Voljč M, Tomažin U, Pirman T. The effect of linseed oils supplementation on performance, fatty acid composition and oxidative status of rabbits. Acta Agric Slov 2011; 98 (2): 119–25.

52. Brenes A, Viveros A, Goni I, et al. Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. Poultry Sci 2008; 87: 307–16.

53. Zhou B, Wu LM, Yang L, Liu ZL. Evidence for a-tocopherol regeneration reaction of green tea polyphenols in SDS micelles. Free Radical Biol Med 2005; 38: 78–84.

54. Yang A, Tume RK. A comparison of beta-carotene-splitting activity isolated from intestinal mucosa of pasture-grazed sheep, goats and cattle. Biochem Mol Biol Int 1993; 30: 209–17.

VPLIV EKSTRUDIRANIH LANENIH SEMEN, VERBASKOZIDA IN VITAMINA E KOT PREHRANSKIH DOPOLNIL NA SERUMSKE BIOKEMIČNE PARAMETRE IN OKSIDATIVNO STANJE V PLAZMI OVC LACAUNE

D. Casamassima, M. Nardoia, M. Palazzo, F. Vizzarri, C. Corino

Povzetek: Določili smo izbrane biokemične parametre v serumu in oksidativno stanje v plazmi ovc Lacaune, hranjenih s krmo z dodanimi ekstrudiranimi lanenimi semeni, verbaskozidom in vitaminom E. Izvedli smo 98 dnevni prehranski poskus na 44 ovcah, ki so bile 40±2 dni po porodu. Živali so bile razdeljene v štiri homogene skupine po enajst živali v skupini. Kontrolna skupina (CON) je bila krmljena z osnovno krmo, ostalim trem skupinam pa smo obrok dopolnili z ekstrudiranimi lanenimi semeni (L), z ekstrudiranimi lanenimi semeni in verbaskozidom (LVB) ali z ekstrudiranimi lanenimi semeni, verbaskozidom in vitaminom E (LVBE). Obroki so bili usklajeni na ravni proteinov in energije, sestavljeni iz 700 g koncentrirane krme in sena *ad libitum*. Kri smo ovcam odvzeli trikrat, in sicer na začetku poskusa (0 d), na sredini (49 d) in na koncu (98 d). V serumu smo določili naslednje parametre: trigliceride, skupni holesterol, holesterol v lipoproteinih z visoko gostoto (HDL) in nizko gostoto (LDL), bilirubin, alanin-aminotransferazo, aspartat-aminotransferazo, tiobarbiturne reaktivne substance (TBARS), reaktivne kisikove metabolite (ROM) ter vitamina A in E. Naši rezultati so pokazali, da so imele živali z dodatkom verbaskozida v skupinah LVB in LVBE boljši serumski profil izmerjenih parametrov, in sicer statistično značilno višji HDL holesterol (p =0,001) in nižjo koncentracijo trigliceridov (p=0,001), celotnega holesterola (p=0,017) , LDL holesterola (p = 0,001) in bilirubina (p=0.035). Ravno tako so imele te živali v krvi več vitamina A (p=0.002) in E (p=0,001) in hkrati manj ROM in TBARS (p=0,001) ter izboljšano oksidativno stanje. Naši rezultati so pokazali, da hrani dodan vitamin E ni vplival na preiskovane parametre, saj ni bilo statističnih razlik med skupinama LVB in LVBE. Tako sklepamo, da ima dodatek verbascozida živalski krmi, ki vsebuje ekstrudirano laneno seme, pozitiven vpliv glede na preiskovane biokemične parametre in oksidativno stanje v krvi ovc.

Ključne besede: ekstrudirano laneno seme, serumski biokemični parametri, verbaskozid, vitamin E, ovce Lacaune