World Rabbit Science

World Rabbit Sci. 2014, 22: 71-81
doi:10.4995/wrs.2014.1183

EFFECT OF DIETARY LINSEED OIL AND *GANODERMA LUCIDUM* OR OLIVE LEAVES SUPPLEMENTATION ON FATTY ACID COMPOSITION AND OXIDATIVE STATUS OF RABBITS

TREBUŠAK T., LEVART A., FRANKIČ T., SALOBIR J., PIRMAN T.

Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale, Slovenia.

Abstract: The aim of the present study was to evaluate the influence of natural antioxidant supplementation on the oxidative stress induced by a high proportion of polyunsaturated fatty acids (PUFA) in rabbit diets. Forty-eight SIKA rabbits (24 male, 24 female) were penned individually and after a 5-d adaptation period divided into 4 homogeneous groups (2.58±0.3 kg). The CONT– group received a diet with 60 g/kg palm fat, while the other 3 groups received a diet with 60 g/kg linseed oil which was either unsupplemented (CONT+) or supplemented with 10 g/kg of *Ganoderma lucidum* (REISHI) or 10 g/kg of olive leaves (OLIVE). Rabbits were euthanised at 102 d of age, 22 d after the start of the experiment. Live weight, weight gain, feed intake and feed conversion rate were recorded. The fatty acid composition of back (*M. longissimus dorsi*) and hind leg muscle (thigh muscle), adipose tissue (abdominal fat) and liver were determined. To evaluate the oxidative status of rabbits, the malondialdehyde (MDA) concentration in the plasma, liver and both muscles was measured. DNA damage in the leukocytes was measured. Linseed oil addition increased the PUFA ratio and decreased the proportion of saturated fatty acids in all tissues (P<0.001), with no detrimental effect on productive performance of rabbits compared to palm fat. Linseed oil also reduced n-6/n-3 PUFA ratio in all tissues (P<0.001). However, linseed oil addition increased the oxidative stress by increasing MDA concentrations in the liver and muscles (P<0.001), but did not affect the plasma MDA concentrations and the extent of DNA damage in the blood leukocytes compared to palm fat. Addition of olive leaves tended to decrease the concentration of MDA in the liver Addition of olive leaves tended to decrease the concentration of MDA in the liver compared to CONT+ (P=0.059), with no effect of *Ganoderma lucidum*.

Key Words: rabbits, olive leaves, *Ganoderma lucidum*, fatty acid composition, oxidative status.

INTRODUCTION

Nowadays people are more concerned about their health and the nutritional value of their food. The beneficial effects of polyunsaturated fatty acids (PUFA) on human health are well known and have been documented by numerous studies (Williams, 2000; Riediger *et al.*, 2009). Populations in the developed world do not consume enough dietary essential nutrients, which include PUFA, especially n-3 PUFA. Deficiency of n-3 PUFA increases the risk of developing various diseases, especially cardiovascular diseases and cancer. Due to modern dietary trends, the interest in obtaining animal products with a higher content of n-3 fatty acids has grown. The fatty acid composition can be improved by different dietary strategies. Because of its high α-linolenic acid (C18:3 n-3) content, flax or linseed oil is a suitable and frequently used plant source of n-3 PUFA.

Rabbit meat is often recommended by nutritionists for its low lipid and cholesterol levels and high PUFA content compared to other meat (Dalle Zotte, 2002), as rabbit diets usually contain alfalfa with high n-3 PUFA content. Western diets are deficient in n-3 PUFA and have excessive amounts of n-6 PUFA. This leads to a very high n-6/n-3 ratio, ranging from 15/1 to 16.7/1, although the optimum ratio to improve human health is thought to be less than 4/1 (Simopoulos, 2002). However, the higher content of PUFA led to a higher susceptibility to

Correspondence: T. Pirman, tatjana.pirman@bf.uni-lj.si. Received *June 2012* - Accepted *September 2013.*
http://dx.doi.org/10.4995/wrs.2014.1183

lipid oxidation. This might cause tissue oxidation, which leads to the formation of free radicals, lipid peroxides, aldehydes (e.g. malondialdehyde, MDA) and further oxidation products which have a negative effect on the dietetic value of fat and are harmful to the organism. Several studies have shown that lipid oxidation is negatively affected by increasing the PUFA n-3 content in rabbit meat. Dietary supplementation with antioxidants is therefore very important to prevent lipid oxidation caused by feeding high levels of PUFA.

The most frequently used antioxidant is vitamin E (α-tocopherol), but recently the interest in using natural antioxidants has increased. Various polyphenols and carotenoids can be included among the natural antioxidants of plant origin analysed in recent studies. Medicinal mushrooms have a proven antioxidant effect and are therefore a suitable supplement to reduce the risk of oxidation. Many studies prove that they contain a number of potential natural antioxidants (Cheung *et al.*, 2003; Cheung and Cheung, 2005; Lindequist *et al.*, 2005) and are also a good source of phenols, flavonoids, carotenes and ascorbic acid (Froufe *et al.*, 2009). They have an established history of use in the traditional medicine, particularly in Asia. In Europe, particularly in the Mediterranean, the olive tree, which today is best known for its olives and olive oil, has always been important in folk medicine. Olive leaves and their extracts are a rich source of phenolic compounds such as oleuropein, hydroxytyrosol and tocopherol (Savournin *et al.*, 2001). Because of the high content of phenolic compounds, olive leaves are also known for their antioxidant activity (Benavente-García *et al.*, 2000, Botsoglou *et al.*, 2010).

The purpose of the present research was to determine the effect of linseed oil addition on performance, fatty acid composition and oxidative status of rabbit, and to evaluate the potential of *Ganoderma lucidum* and olive leaves in prevention of oxidation.

MATERIALS AND METHODS

All procedures were performed according to current legislation on animal experimentation in Slovenia. Animals used in this experiment were reared and euthanised at the Department of Animal Science, Biotechnical Faculty (Ljubljana, Slovenia).

Animals and diets

Forty-eight SIKA rabbits (24 male, 24 female; average initial body weight 2579±299 g), were randomly divided into 4 groups after 5-d adaptation period (all groups receiving commercial diet) and assigned to 4 different dietary treatments for 22 d (12 rabbits/treatment). Animals had free access to pelleted feed and water (nipple drinkers) and were housed individually in the wire cages (45.5×37.5×30 cm). The CONT– group received a diet with 60 g/kg palm fat, whereas the other 3 groups received a diet with 60 g/kg linseed oil, either unsupplemented (CONT+) or supplemented with 10 g/kg of *Ganoderma lucidum* (REISHI) or 10 g/kg of olive leaves (OLIVE). *Ganoderma lucidum* or reishi mushroom was added to the feed as the product Galimmun® (Institute of Natural Sciences, Ljubljana, Slovenia) which contains milled fruiting bodies and mycelia of organically produced mushrooms. The immunologically active components are β-glucans (155 g/kg) and triterpenes (16.8 g/kg) (Institute of Natural Sciences, Ljubljana, Slovenia). Olive leaves were picked at a local olive oil-press (Oljarna Babič, Marezige, Slovenia), dried, milled and stored in a dry and dark place. Chemical composition of *Ganoderma lucidum* and olive leaves was determined in our laboratory by standard procedures: dry matter (AOAC Official method 934.01), crude protein (AOAC Official method 984.13), crude fat (AOAC Official method 920.39), crude fibre (AOAC Official method 978.10) and crude ash (AOAC Official method 942.05) (AOAC, 2000) and their antioxidant potential and vitamin E concentration are presented in Table 1. The ingredients, chemical and fatty acid composition of the diets are presented in Table 2. As expected, the fatty acid composition of the diets differed according to the ingredients. Diets with linseed oil (CONT+, REISHI, OLIVE) had higher proportion of n-3 and n-6 PUFA, total PUFA and monounsaturated fatty acids (MUFA), and lower proportion of saturated fatty acids (SFA) compared to the diet with palm fat (CONT–). The linseed oil diets had higher proportion of linoleic acid (C18:2 n-6) and α-linolenic acid (C18:3 n-3) and lower n-6/n-3 ratio compared to the diet with palm fat (0.55 *vs.* 3.16).

Table 1: *In vitro* antioxidant potential determined by photochemiluminiscence, vitamin E concentration and chemical composition of used supplements.

	G. lucidum (Galimmun®. REISHI)	Olive leaves (OLIVE)
ACW (mg/kg)	521.0	110981.0
ACL (mg/kg)	15.4	720.0
Vitamin E (mg/kg)		
α-tocopherol	n.d.	195.1
γ-tocopherol	2.1	2.8
Chemical composition (g/kg DM)		
Dry matter (DM; g/kg)	979	939
Crude protein	183	119
Crude fat	12	31
Crude fibre	201	240
Crude ash	44	74

ACW: water soluble antioxidants; mg/kg of gallic acid equivalents; ACL: lipid soluble antioxidants; mg/kg of trolox equivalents. n.d.: not detected.

Experimental procedure and sample collection

Each day, animals received a weighed daily meal and the residue from the previous day was weighed and discarded. Body weight was recorded each week during the experimental period and just before slaughter. After 22 d of treatment, rabbits were euthanised at 102 d of age, by electric stunning and exsanguination. Blood samples, for the purpose of measuring MDA concentration in the plasma and DNA fragmentation of blood leukocytes, were collected into 6 mL evacuated tubes containing $EDTAK_2$ anticoagulant (367864, BD-Plymouth, UK). Carcasses were then prepared by removing skin, distal part of the tail, fore and hind legs, head, gastrointestinal and urogenital tract. The whole liver was taken, weighed and stored at –70 °C until analyses were performed. After 24 h chilling at 4 °C, samples of hind leg muscle (thigh muscle), back muscle (*M. longissimus dorsi*) and adipose tissue (abdominal fat) were taken and stored at –70 °C until analyses were performed.

Determination of vitamin E (α- and γ-tocopherol) and antioxidant capacity

Concentrations of vitamin E in the supplements (REISHI and OLIVE) were measured according to the methodology of Abidi and Mounts (1997) and Rupérez *et al.* (2001) and quantification of vitamin E isomers was performed using an external standard (Calbiochem® tocopherol set; alpha, beta, gamma and delta, 5 point calibration curve). The samples were analysed by reverse-phase high performance liquid chromatography (HPLC), using a Luna 5u PFP(2) column (100A 250×4.6 mm; Phenomenex Inc., Torrance, CA, USA) and Waters 474 scanning fluorescence detector. The mobile phase consisted of 95% methanol and 5% water and the mobile phase rate was 1.2 mL/min. The results of the analysis were evaluated using the Millenium^{32} Chromatography Manager (Waters, Milford, MA, USA) program.

The antioxidant capacity of the lipid soluble (ACL) and water soluble (ACW) compounds in the supplements was measured using the photochemiluminescence method by PhotoChem® (Analytik Jena, Jena, Germany) and presented as Trolox equivalents (ACL) or gallic acid equivalents (ACW). Samples were extracted with methanol and the extracts obtained were analysed according to ACL-Kit or ACW-Kit protocol (Analytik Jena, Jena, Germany).

Determination of fatty acid composition

The fatty acid composition of diets, muscles, adipose tissue and liver were analysed using a gas chromatographic method after the *in situ* transesterification of lipids. Each sample was analysed in duplicate. Methyl esters of fatty acids were prepared according to the procedure of Park and Goins (1994). A brief summary of the procedure is as follows: frozen sample was homogenised (Grindomix Homogenizer, Retsch Gmbh & Co, Haan, Germany) and approximately 0.5-0.7 g (diets, muscles, liver) or 0.1 g (adipose tissue) of the homogenised sample was weighed directly in a tube with stopper and mixed with 3 mL 0.5 M sodium hydroxide in methanol and 0.3 mL methylene chloride. *In situ* transesterification was performed by heating samples at 90 °C for 10 min in the closed tube. After cooling, 3 mL of 14% boron trifluoride in the methanol was added and heating at 90 °C was continued for 10 min in the closed tube. Samples were cooled and the fatty acid methyl esters (FAMEs) were extracted into 1 mL hexane. Analysis of FAMEs was performed by gas chromatography using an Agilent 6890 series gas chromatograph (Agilent

Table 2: Ingredients, chemical composition and fatty acid composition of experimental diets.

	CONT–	CONT+	REISHI	OLIVE
Ingredients (g/kg)				
Alfalfa	458.3	458.3	448.3	448.3
Barley	130.0	130.0	130.0	130.0
Sunflower meal	210.0	210.0	210.0	210.0
Hay meal	100.0	100.0	100.0	100.0
Rapeseed oil	10.0	10.0	10.0	10.0
Palm fat	60.0	0	0	0
Linseed oil	0	60.0	60.0	60.0
Ganoderma lucidum	0	0	10.0	0
Olive leaves	0	0	0	10.0
Methionine	0.5	0.5	0.5	0.5
Lysine	2.0	2.0	2.0	2.0
Vitamin-mineral mix [1]	5.0	5.0	5.0	5.0
Lignobond [2]	20.0	20.0	20.0	20.0
Salt	4.2	4.2	4.2	4.2
Chemical composition (g/kg DM)				
Dry matter (g/kg)	933	912	924	922
Crude protein	191	196	196	193
Crude fat	116	95	92	93
Crude fibre	244	250	247	246
Crude ash	74	76	77	76
Main fatty acids (% of the total fatty acids)				
C12:0	0.20	0.04	0.04	0.04
C14:0	0.89	0.12	0.12	0.12
C16:0	35.81	8.00	8.06	8.07
C 16:1 n-7	0.09	0.18	0.18	0.18
C18:0	41.73	4.00	3.98	3.99
$\sum$ C18:1	7.75	23.48	23.70	23.56
C18:2 n-6	9.01	22.01	22.20	21.92
C18:3 n-3	2.78	40.23	39.77	40.21
C 22:6 n-3	0.08	0.10	0.09	0.10
$\sum$ SFA	80.08	13.48	13.53	13.56
$\sum$ MUFA	8.05	24.14	24.36	24.22
$\sum$ PUFA	11.86	62.38	62.11	62.22
$\sum$ n-3 PUFA	2.85	40.33	39.86	40.31
$\sum$ n-6 PUFA	9.01	22.05	22.25	21.92
n-6/n-3 PUFA	3.16	0.55	0.56	0.54

CONT–: 60 g/kg palm fat in a diet; CONT+: 60 g/kg linseed oil in a diet; REISHI: 60 g/kg linseed oil in a diet with addition of 10 g/kg *Ganoderma lucidum*; OLIVE: 60 g/kg linseed oil in a diet with addition of 10 g/kg olive leaves. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

[1] Vitamin-mineral mix (0.5 %) (mg/kg diet): vitamin A (33,333 IU), vitamin D_3 (80,000 IU), vitamin E (60 IU), vitamin K_3 (4 mg), vitamin B_1 (2 mg), vitamin B_2 (7.5 mg), vitamin B_3 (50 mg), vitamin B_5 (20 mg), vitamin B_6 (2 mg), vitamin B_{12} (10 mg), folic acid (5 mg), biotin (5 g), choline (333 mg), Fe (156 mg), Cu (320 mg), Mn (161 mg), Co (100 mg), I (2.95 mg), Se (15 mg), Zn (125 mg).

[2] pellet binder

Proportions of following fatty acids were below the detection limit (LOD = 0.01%): 20:5 n-3, 20:5 n-6, 22:5 n-3.

Technologies, Wilmington, DE, USA) equipped with an Agilent 7683 Automatic Liquid Sampler, a split injector, a flame ionisation detector and a fused silica capillary column Omegawax 320 (Supelco, USA). The injection volume was 1 µL. The chromatograms were evaluated using Agilent GC Chem Station software. Separated FAMEs were identified by retention time. Results are expressed as a percentage of the total fatty acids.

Malondialdehyde (MDA) determination

The methodology of Wong *et al.* (1987) modified by Chirico (1994) and Fukunaga *et al.* (1995) was used to measure the concentration of MDA in the blood plasma using HPLC and the quantification of MDA was performed using

external standard (TEP, 5 point calibration curve). A brief summary of the procedure is as follows: 100 µL of the sample was mixed with 100 µL of 0.44 M phosphoric acid (H_3PO_4) and 10 µL of 0.2% ethanolic butylhydroxytoluene (BHT) in the Eppendorf microcentrifuge tubes and left for 15 min before adding 300 µL ethanol and then centrifuging (15000 *g*, for 15 min at 4 °C). The supernatant (350 µL) was mixed with 1.5 mL of 0.44 M H_3PO_4, 0.5 mL of 0.6% thiobarbituric acid (TBA) and 0.9 mL of Milli Q deionised water in a tube with stopper and heated at 90 °C for 60 min. After cooling, the samples were filtered through Millipore filters (pore size 0.22 µm) into auto sampler vials. MDA concentration in the liver and muscle samples was determined following the method of Vila *et al.* (2002) with minor modifications. Briefly, frozen samples were homogenised (Grindomix Homogenizer, Retsch Gmbh & Co, Haan, Germany) and approximately 0.3 g of the homogenised sample was mixed with 1.5 mL of 2.5% trichloroacetic acid (TCA) in the Eppendorf microcentrifuge tubes, left for 10 min and then centrifuged (15000 *g*, for 15 min at 4 °C). One mL of supernatant was mixed with 1.5 mL of 0.6% TBA and 1 mL of Milli Q deionised water in a tube with stopper and heated at 90 °C for 60 min. After the samples were cooled, they were filtered through Millipore filters (pore size 0.22 µm) into auto sampler vials. A Waters Alliance 2690 (Waters, Milford, MA) equipped with Waters 474 scanning fluorescence detector was used to determine plasma, liver and muscle MDA. For separation, a reversed-phase HPLC chromatography column (HyperClone 5u ODS (C_{18}) 120 A, 4.6×150 mm 5 micron; Phenomenex Inc., USA) and a C_{18} ODS guard column (4×30 mm; Phenomenex Inc., USA) were used. The mobile phase consisted of 65% 50 mmol/L KH_2PO_4 buffer (pH 6.9) and 35% methanol. The mobile phase flow rate was 1.0 mL/min. The results of the analysis were evaluated using the Millenium^{32} Chromatography Manager (Waters, Milford, MA, USA) program.

Leukocyte isolation and comet assay

DNA fragmentation was examined in the leukocytes isolated from fresh rabbit blood. Leukocytes were isolated from the blood samples according to a procedure described by Johnstone and Thorpe (1990). Comet assay was performed according to Singh *et al.* (1988), with slight modifications as described by Rezar *et al.* (2003). An Olympus CH 50 epifluorescence microscope at 200× magnification was used for the examination of leukocyte nuclei in the microgels (100W Hg lamp, excitation filter of 480-550 nm and barrier filter of 590 nm). The images were captured by a Hamamatsu Orca 1 CCD camera, analysed, and the nuclear DNA damage was estimated by the Comet 5 dedicated computer program (Single Cell Gel Electrophoresis, Kinetic Imaging Ltd., 2000). DNA damage was evaluated as % of DNA in the tail of the comet and as Olive tail moment (OTM). OTM is defined as the product of the amount of DNA in the tail and the mean distance of migration in the tail (higher values represent a higher rate of DNA damage) (Olive *et al.*, 1992).

Statistical analysis

The data were analysed using the General Linear Models (GLM) procedure of the SAS/STAT module (SAS 8e, 2000; SAS Inc., Cary, NC, USA), taking the diet into consideration as the only main effect. Differences between groups were determined on the basis of Tukey's multiple comparisons test. Unless stated otherwise, a least significant difference of 0.05 was used to separate treatment means. Results in the tables are presented as least square means (LSM)±SEM with *P*-values.

RESULTS AND DISCUSSION

During the experiment, 2 animals (females from the CONT+ and OLIVE group) had health problems (feet blisters) and were eliminated from the experiment. Growth performances of rabbits were normal according to age (Table 3), indicating that the addition of olive leaves or *G. lucidum* had no adverse effect on the growth rate of rabbits. Dietary inclusion of linseed oil did not affect growing rabbit performance, as other authors have previously reported (Bianchi *et al.*, 2009; Casado *et al.*, 2013)

Fatty acid composition of tissues and oxidative status of rabbits

Diets supplemented with linseed oil led to a higher proportion of total PUFA, due to a reduction in total SFA levels in the muscle, adipose tissue and liver (Tables 4, 5, 6 and 7), and also caused a reduction of total MUFA levels in the

Table 3: Effect of dietary linseed oil and *Ganoderma lucidum* or olive leaves supplementation on body weight (BW), weight gain and diet intake.

	CONT–	CONT+	REISHI	OLIVE	SEM[1]	*P*-value
Initial BW (g)	2574	2589	2563	2595	93.3	0.99
Final BW (g)	3235	3362	3303	3396	102.7	0.69
Weight gain (g/d)	28.8	33.6	32.2	34.8	2.19	0.22
Feed intake (g/d)	168	179	169	175	5.46	0.48
FCR (g/g)	6.09	5.66	5.39	5.18	0.37	0.32

CONT–: 60 g/kg palm fat in a diet; CONT+: 60 g/kg linseed oil in a diet; REISHI: 60 g/kg linseed oil in a diet with addition of 10 g/kg *Ganoderma lucidum*; OLIVE: 60 g/kg linseed oil in a diet with addition of 10 g/kg olive leaves.

FCR: feed conversion rate.

[1]SEM: Standard error of the means (n=12).

liver (Table 6). In all the tissues, the linseed oil addition increased ($P<0.001$) the proportion of the α-linolenic acid and some other n-3 PUFA (Tables 4, 5, 6 and 7) compared to CONT– group. Due to these differences, the higher content of n-3 PUFA in all 3 diets containing linseed oil led to a lower ($P<0.001$) n-6/n-3 PUFA ratio in all the tissues. Similar results, increased proportion of α-linolenic acid and decreased n-6/n-3 PUFA ration, were obtained in the other studies where ground or extruded linseed was added to the diet, compared to control (Bernardini *et al.*, 1999, Kouba *et al.*, 2008). Linseed addition also increased the long chain n-3 PUFA contents in the meat and consecutively caused a significant decrease in the n-6/n-3 PUFA ratio (Kouba *et al.*, 2008). Decreased n-6/n-3 PUFA ratio in rabbit muscle, adipose tissues and liver can provide healthier food for human consumption. Intake of such food contributes to a better ratio of n-6/n-3 PUFA in the human diet and thus contributes to improved human health.

The addition of *G. lucidum* (in both muscles) and olive leaves (only in the leg muscle) increased ($P<0.05$) the proportion of docosahexaenoic acid (C22:6 n-3; DHA) compared to CONT– group (Tables 4 and 5); otherwise the addition of olive leaves and *G. lucidum* did not influence fatty acid composition of tissues. DHA is anti inflammatory fatty acid and may also reduce the risk of heart and circulatory disease by decreasing blood thickness and lowering blood levels of triglycerides (Simopoulos, 2009) and consuming meat with increased amounts of DHA improves human health. The proportion of linoleic acid only increased significantly in the adipose tissue of rabbits feed on linseed oil supplemented

Table 4: Effect of dietary linseed oil and *Ganoderma lucidum* or olive leaves supplementation on fatty acid composition of back muscle (*M. longissimus dorsi*) (% of total fatty acids).

	CONT–	CONT+	REISHI	OLIVE	SEM[1]	*P*-value
C12:0	0.15	0.092	0.14	0.11	0.02	0.060
C14:0	1.86 ^{a}	1.44 ^{b}	1.28 ^{b}	1.40 ^{b}	0.10	<0.001
C16:0	23.21 ^{a}	17.31 ^{b}	17.66 ^{b}	17.62 ^{b}	0.34	<0.001
C16:1 n-7	3.48	2.64	2.41	2.55	0.45	0.32
C18:0	10.48 ^{a}	6.02 ^{b}	6.84 ^{b}	6.49 ^{b}	0.33	<0.001
∑ C18:1	23.38	23.50	22.76	23.12	0.30	0.29
C18:2 n-6	23.91	24.25	24.30	24.17	0.50	0.94
C18:3 n-3	3.15 ^{a}	14.68 ^{b}	12.50 ^{b}	13.48 ^{b}	0.74	<0.001
C20:4 n-6	4.12	3.43	4.44	3.91	0.39	0.31
C20:5 n-3	0.14 ^{a}	0.39 ^{b}	0.45 ^{b}	0.41 ^{b}	0.04	<0.001
C22:5 n-3	0.63 ^{a}	1.11 ^{b}	1.36 ^{b}	1.23 ^{b}	0.11	<0.001
C22:6 n-3	0.11 ^{a}	0.15 ^{ab}	0.19 ^{b}	0.17 ^{ab}	0.02	0.005
∑ SFA	38.52 ^{a}	27.50 ^{b}	28.98 ^{b}	28.43 ^{b}	0.53	<0.001
∑ MUFA	27.80	27.06	26.11	26.60	0.74	0.40
∑ PUFA	33.68 ^{a}	45.44 ^{b}	44.91 ^{b}	44.97 ^{b}	0.85	<0.001
∑ n-3 PUFA	4.07 ^{a}	16.52 ^{b}	14.68 ^{b}	15.50 ^{b}	0.68	<0.001
∑ n-6 PUFA	29.59	28.90	30.21	29.45	0.65	0.55
n-6/n-3 PUFA	7.28 ^{a}	1.81 ^{b}	2.11 ^{b}	1.96 ^{b}	0.13	<0.001

CONT–: 60 g/kg palm fat in a diet; CONT+: 60 g/kg linseed oil in a diet; REISHI 60 g/kg linseed oil in a diet with addition of 10 g/kg *Ganoderma lucidum*; OLIVE: 60 g/kg linseed oil in a diet with addition of 10 g/kg olive leaves.

^{a,b} Means in a row with different superscripts are significantly different ($P<0.05$); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

[1] SEM: Standard error of the means (n=12).

Table 5: Effect of dietary linseed oil and *Ganoderma lucidum* or olive leaves supplementation on fatty acid composition of hind leg muscle (thigh muscle) (% of total fatty acids).

	CONT –	CONT +	REISHI	OLIVE	SEM[1]	*P*-value
C12:0	0.20	0.16	0.20	0.18	0.02	0.21
C14:0	1.92 [a]	1.47 [b]	1.35 [b]	1.42 [b]	0.08	<0.001
C16:0	22.69 [a]	16.52 [b]	16.54 [b]	16.87 [b]	0.34	<0.001
C16:1 n-7	2.92	2.09	1.90	1.83	0.32	0.07+
C18:0	10.86 [a]	6.09 [b]	7.80 [b]	6.56 [b]	0.38	<0.001
∑ C18:1	23.15	23.01	22.40	22.71	0.34	0.38
C18:2 n-6	27.83	28.06	29.00	28.38	0.54	0.42
C18:3 n-3	3.99 [a]	16.26 [b]	14.69 [b]	15.32 [b]	0.67	<0.001
C20:4 n-6	1.85	1.60	1.96	1.78	0.14	0.31
C20:5 n-3	0.061 [a]	0.18 [b]	0.19 [b]	0.18 [b]	0.01	<0.001
C22:5 n-3	0.33 [a]	0.59 [b]	0.68 [b]	0.64 [b]	0.05	<0.001
C22:6 n-3	0.058 [a]	0.083 [ab]	0.10 [b]	0.091 [b]	0.008	0.003
∑ SFA	37.97 [a]	26.37 [b]	27.26 [b]	27.29 [b]	0.50	<0.001
∑ MUFA	26.82	25.85	24.99	25.19	0.67	0.19
∑ PUFA	35.21 [a]	47.78 [b]	47.75 [b]	47.52 [b]	0.73	<0.001
∑ n-3 PUFA	4.50 [a]	17.26 [b]	15.81 [b]	16.43 [b]	0.68	<0.001
∑ n-6 PUFA	30.68	30.48	31.92	31.06	0.65	0.40
n-6/n-3 PUFA	6.82 [a]	1.81 [b]	2.09 [b]	1.95 [b]	0.13	<0.001

CONT–: 60 g/kg palm fat in a diet; CONT+: 60 g/kg linseed oil in a diet; REISHI: 60 g/kg linseed oil in a diet with addition of 10 g/kg *Ganoderma lucidum*; OLIVE: 60 g/kg linseed oil in a diet with addition of 10 g/kg olive leaves.

[a, b] Means in a row with different superscripts are significantly different ($P<0.05$); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

[1] SEM: Standard error of the means (n=12).

with REISHI (Table 7). To our knowledge, there are no studies of the effect of *G. lucidum* on the fatty acid composition, although there are a few dealing with olive leaves. These studies also showed that addition of olive leaves in diet had no effect on fatty acid composition (Paiva-Martins *et al.*, 2009).

Table 6: Effect of dietary linseed oil and *Ganoderma lucidum* or olive leaves supplementation on fatty acid composition of liver (% of total fatty acids).

	CONT –	CONT +	REISHI	OLIVE	SEM[1]	*P*-value
C12:0	0.041 [a]	0.021 [b]	0.035 [ab]	0.033 [ab]	0.005	0.030
C14:0	0.35 [a]	0.18 [b]	0.18 [b]	0.19 [b]	0.03	<0.001
C16:0	17.23 [a]	12.06 [b]	12.18 [b]	12.42 [b]	0.31	<0.001
C16:1 n-7	0.63 [a]	0.45 [b]	0.40 [b]	0.43 [b]	0.05	0.003
C18:0	24.55	23.74	23.44	23.56	0.36	0.12
∑ C18:1	13.34 [a]	11.00 [b]	11.15 [b]	10.83 [b]	0.38	<0.001
C18:2 n-6	30.50	31.19	30.88	31.31	0.29	0.20
C18:3 n-3	1.70 [a]	7.04 [b]	7.65 [b]	6.92 [b]	0.27	<0.001
C20:4 n-6	7.12	7.10	7.07	7.05	0.19	0.99
C20:5 n-3	0.15 [a]	0.46 [b]	0.45 [b]	0.43 [b]	0.03	<0.001
C22:5 n-3	0.44 [a]	1.51 [b]	1.50 [b]	1.52 [b]	0.06	<0.001
C22:6 n-3	0.17 [a]	0.61 [b]	0.56 [b]	0.59 [b]	0.03	<0.001
∑ SFA	43.29 [a]	37.75 [b]	37.60 [b]	37.96 [b]	0.38	<0.001
∑ MUFA	14.40 [a]	11.85 [b]	11.96 [b]	11.68 [b]	0.42	<0.001
∑ PUFA	42.31 [a]	50.40 [b]	50.45 [b]	50.36 [b]	0.25	<0.001
∑ n-3 PUFA	2.54 [a]	9.96 [b]	10.49 [b]	9.83 [b]	0.31	<0.001
∑ n-6 PUFA	39.75	40.43	39.96	40.52	0.32	0.26
n-6/n-3 PUFA	15.81 [a]	4.11 [b]	3.88 [b]	4.17 [b]	0.31	<0.001

CONT–: 60 g/kg palm fat in a diet; CONT+: 60 g/kg linseed oil in a diet; REISHI: 60 g/kg linseed oil in a diet with addition of 10 g/kg *Ganoderma lucidum*; OLIVE: 60 g/kg linseed oil in a diet with addition of 10 g/kg olive leaves.

[a, b] Means in a row with different superscripts are significantly different ($P<0.05$); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

[1] SEM: Standard error of the means (n=12).

Table 7: Effect of dietary linseed oil and *Ganoderma lucidum* or olive leaves supplementation on fatty acid composition of adipose tissue (% of total fatty acids).

	CONT–	CONT+	REISHI	OLIVE	SEM[1]	*P*-value
C12:0	0.19	0.15	0.18	0.16	0.02	0.43
C14:0	1.75 [a]	1.24 [b]	1.22 [b]	1.25 [b]	0.06	<0.001
C16:0	24.81 [a]	15.86 [b]	15.76 [b]	16.12 [b]	0.37	<0.001
C16:1 n-7	2.18	1.73	1.51	1.51	0.20	0.06
C18:0	14.06 [a]	5.43 [b]	5.71 [b]	5.63 [b]	0.32	<0.001
∑ C18:1	23.66 [a]	24.71 [b]	24.26 [ab]	24.41 [ab]	0.25	0.030
C18:2 n-6	25.72 [a]	27.23 [a]	28.95 [b]	27.92 [a]	0.60	0.003
C18:3 n-3	4.21 [a]	20.31 [b]	18.93 [b]	19.61 [b]	0.72	<0.001
C20:4 n-6	0.14	0.14	0.14	0.13	0.005	0.42
C20:5 n-3	0.001 [a]	0.03 [b]	0.03 [b]	0.03 [b]	0.002	<0.001
C22:5 n-3	0.06 [a]	0.11 [b]	0.11 [b]	0.11 [b]	0.005	<0.001
∑ SFA	42.72 [a]	24.41 [b]	24.76 [b]	24.96 [b]	0.59	<0.001
∑ MUFA	26.58	27.13	26.42	26.56	0.41	0.61
∑ PUFA	30.70 [a]	48.47 [b]	48.83 [b]	48.49 [b]	0.67	<0.001
∑ n-3 PUFA	4.36 [a]	20.65 [b]	19.26 [b]	19.96 [b]	0.72	<0.001
∑ n-6 PUFA	26.30 [a]	27.79 [ab]	29.52 [b]	28.49 [ab]	0.62	0.004
n-6/n-3 PUFA	6.02 [a]	1.40 [b]	1.58 [b]	1.45 [b]	0.10	<0.001

CONT–: 60 g/kg palm fat in a diet; CONT+: 60 g/kg linseed oil in a diet; REISHI: 60 g/kg linseed oil in a diet with addition of 10 g/kg *Ganoderma lucidum*; OLIVE: 60 g/kg linseed oil in a diet with addition of 10 g/kg olive leaves. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. Proportions of following FAs were below the detection limit (LOD=0.01%): 20:5 n-6.

[a, b] Means in a row with different superscripts are significantly different ($P<0.05$).

[1] SEM: Standard error of the means (n=12).

Oxidative stress is caused by providing large amounts of PUFA from linseed oil and was expressed by increasing MDA concentration in the liver and muscles, but did not affect the leukocyte DNA damage and MDA concentration in the plasma (Table 8) or in the urine (8.19±0.89 nmol/mL in average, not presented). The level of vitamin E in the diet, which is one of the antioxidants already present in the diets, seems to be efficient in protection of oxidative damage to plasma (MDA concentration and leukocytes DNA damage), since there were no differences among groups, but in the liver and muscles neither vitamin E nor supplements (*G. lucidum* or olive leaves) were successful, as the antioxidant levels in the diets were not high enough to protect lipid oxidation in liver and meat. All 3 groups with linseed oil high in n-3 PUFA, showed higher ($P<0.001$) levels of MDA in the muscles compared to CONT– group (Table 8). Similar results were obtained in the liver, except for the OLIVE group, which did not differ from CONT– group. Tres *et al.* (2009) found out that increasing vegetable fat (up to 30 g/kg) in the diet led to higher susceptibility to oxidation in rabbit plasma, lipid hydroperoxide value of rabbit plasma increased, as did MDA concentration in the meat, but not the MDA concentration in the plasma, which was below the detection limit (13 µg MDA/L of plasma) in all groups. In pigs and chicken the higher consumption of PUFA causes lipid oxidation in the plasma. Addition of 50 g/kg of linseed oil to the pigs' diet caused a twofold higher MDA concentration in the plasma compared to control (Rezar *et al.*, 2003), while the addition of 75 g/kg of linseed oil in chickens' diet increased the MDA concentration fivefold (Voljč *et al.*, 2011).

Lipid oxidation is undesirable from the nutritional point of view, shortens the shelf-life of meat and meat products (Xiccato, 1999; Wood *et al.*, 2003; Bianchi *et al.*, 2006) and could also have negative effects on animal health. Oxidative stress is defined as a persistent imbalance between antioxidants and pro-oxidants in favour of the latter and it can be reduced if we provide enough antioxidants to prevent the formation of free radicals. The extent of DNA damage in the blood leukocytes is presented in Table 8, but no differences among groups were recorded, although the addition of linseed oil increased the oxidative stress by increasing MDA concentration in the liver and muscles. Several studies have shown that addition of linseed oil or some other vegetable fat to the diet had a beneficial effect on fatty acid composition of rabbit meat. In combination with vitamin E, which is known to be a natural antioxidant that protects cell membranes against oxidative damage, it prevents oxidation processes and improves the oxidative stability of meat (Bielanski and Kowalska, 2008; Zsédely *et al.*, 2008). Plasma and liver fatty acids composition also reflected feed's fatty acid profile (Tres *et al.*, 2009).

Table 8: Effect of dietary linseed oil and *Ganoderma lucidum* (reishi) or olive leaves supplementation on MDA concentration in plasma, liver and muscles and leukocytes DNA damage presented as OTM and percentage of DNA in the tail.

	CONT–	CONT+	REISHI	OLIVE	SEM[1]	*P*-value
MDA concentration						
Plasma MDA (nmol/mL)	0.14	0.14	0.15	0.13	0.01	0.78
Liver MDA (nmol/g)	1.05 [a]	2.20 [b]	1.79 [b]	1.50 [ab]	0.19	<0.001
Muscle MDA (nmol/g)						
hind leg muscle	3.42 [a]	12.13 [b]	10.79 [b]	11.18 [b]	1.06	<0.001
back muscle	0.67 [a]	3.69 [b]	2.61 [b]	2.53 [b]	0.45	<0.001
Leukocytes DNA damage						
OTM	1.98	2.37	1.95	2.48	0.33	0.59
Tail DNA (%)	9.98	10.42	9.99	10.50	0.77	0.94

CONT–: 60 g/kg palm fat in a diet; CONT+: 60 g/kg linseed oil in a diet; REISHI: 60 g/kg linseed oil in a diet with addition of 10 g/kg *Ganoderma lucidum*; OLIVE: 60 g/kg linseed oil in a diet with addition of 10 g/kg olive leaves. MDA: malondialdehyde; OTM: Olive tail moment.

[a, b] Means in a row with different superscripts are significantly different ($P<0.05$)

[1] SEM: Standard error of the means (n=12).

In our study, *G. lucidum* and olive leaves did not totally prevent oxidation, but the protection was shown by the increased amount of DHA in the muscles. DHA is an anti-inflammatory, anti-atherogenic, anti-carcinogenic, etc fatty acid and its higher content in the meat had a beneficial effect on human health (Docosahexaenoic acid, 2009). Higher level supplementation of both supplements, in some published data, shows some antioxidant properties. According to Jones and Janardhanan (2000) methanol extracts of *G. Lucidum* possess *in vitro* antioxidant activity. Similar results were obtained by Lakshmi *et al.* (2003) with an ethanol extract of the mycelium of *G. lucidum* which inhibited Fe^{2+}-induced peroxidation of lipid in rat liver (50% inhibition) and maximally inhibited (37%) croton oil-induced peroxidation on mouse skin. Lee *et al.* (2001) tested the amino-polysaccharide fraction from *G. lucidum* for the ability to protect against oxidative damage induced by reactive oxygen species and found that this fraction significantly inhibited iron- or iron plus ascorbic acid-induced lipid peroxidation in the rat brain homogenates 64.2% at highest concentration (2 mg/mL) and showed a dose-dependent inactivation of hydroxyl radicals and superoxide anions. Antioxidant activity of mushrooms is mainly related to their content in phenolic compounds (Froufe *et al.*, 2009), which is also typical for olives, olive oil and leaves (Benavente-García *et al.*, 2000). Oleuropein, the major constituent of the secoiridoid family in the olive trees, has been shown to be a potent antioxidant. It is also the active component of olive leaves. The antioxidant activity of oleuropein was studied using *in vivo* methods. Oleuropein, obtained from olive leaves, might inhibit hyperglycemia and oxidative stress induced by diabetes in rabbits (Al-Azzawie and Alhamdani, 2006). Sixteen weeks of treatment of diabetic rabbits with 20 mg/kg body weight of oleuropein decreased the level of MDA along with blood glucose to the level of normal control rabbits. The level of oleuropein in the OLIVE diet in our experiment was 5 times lower than in the cited work (approximately 4 mg/kg body weight, calculated according to Tayoub *et al.*, 2012), although the experiment duration was shorter and the way the potential antioxidants were added was different. In our study, we used ground olive leaves and mushrooms, while others use different extracts, which showed better effects. However, our experiment also revealed a certain trend in the terms of antioxidant protection, which could be better if we used extracts or higher levels of supplementation.

CONCLUSION

The overall fatty acid composition was influenced by dietary linseed oil, with higher amounts of PUFA, especially n-3 PUFA, lower amount of SFA and no effect on MUFA and n-6 PUFA. However, the addition of linseed oil led to a higher susceptibility to lipid oxidation in the muscle and liver. *G. lucidum* had an additional effect on the increase of DHA in muscles, especially in back muscle, but was less effective in prevention of oxidation. Addition of olive leaves as a natural antioxidant slightly reduced the concentration of MDA, but did not completely prevent oxidation. DHA in the muscles increased slightly, suggesting that the level of supplementation of olive leaves and *G. lucidum* should be higher to achieve a more observable direct effect on oxidative stability.

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