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Effect of hemp seed oil supplementation on plasma lipid profile, liver function, milk fatty acid, cholesterol, and vitamin A concentrations in Carpathian goats

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ABSTRACT: The aim of this study was to evaluate the effect of dietary supplementation with hemp seed oil (HSO) on the lipid metabolism, through the plasma lipid profile, liver function and concentrations of fatty acids (FA), cholesterol, and vitamin A in goat milk. Ten Carpathian goats were divided into two groups: one was fed the control diet (C) composed of alfalfa hay and concentrate, and the other was fed C diet supplemented with 93 g/day of HSO (4.7% of dry matter intake) for 31 days. The HSO supplementation did not modify plasma cholesterol, triglyceride or phospholipid concentrations, or the activities of alanine aminotransferase or γ-glutamyltransferase, but plasma total lipid concentration was increased. HSO supplementation had no effect on milk yield, but it increased milk fat and protein contents. HSO supplementation markedly altered milk FA composition, but had no effect on milk cholesterol or vitamin A concentrations. Changes in milk FA composition corresponded to a decrease in saturated *de novo* synthesized FA (10:0-16:0) and an increase in 4:0, 18:0, and polyunsaturated FA concentrations. Also, HSO supplementation strongly increased *cis*-9,*trans*-11-CLA and also *trans*-18:1 concentrations in milk fat. These findings suggest that HSO can be used to modify milk FA content with a putative positive effect on human health, without adversely affecting goat performance or health, except for a potential hyperlipidemic effect.

Keywords: dairy goats; plant oil; milk liposoluble components; lipid metabolism

INTRODUCTION

Goat livestock in Romania increased by 56% over the period 2005–2012, achieving a milk production of 229 000 t, the fourth largest production among the European countries in 2011 (Institut de l'Elevage 2013). The increase in Romanian goat milk production in recent years is due to the increasing consumer demand owing to the dietary value and putative health effects of goat milk (Haenlein 2004). Milk fat is one of the main components in the nutritional quality of goat milk, which contains fatty acids (FA) and fat-soluble vitamins with potentially positive effects on hu-

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man health. In particular, goat milk has high concentrations of caproic (6:0), caprylic (8:0), and capric (10:0) acids, known to exhibit antiobesity and antidiabetic properties (Nagao and Yanagita 2010). Butyric acid (4:0) and the major isomer of conjugated linoleic acid (CLA), *cis*-9,*trans*-11, have shown anticarcinogenic effects both *in vitro* and *in vivo*, and vitamin A may exert a major role in cell growth and differentiation (Mills et al. 2011; Plozza et al. 2012). By contrast, some saturated FA (SFA) (12:0, 14:0, 16:0), *trans*-18:1 FA, and cholesterol, if consumed in excess, have been associated with an increased cardiovascular disease risk (Shingfield et al. 2008; Larsson et al. 2012). Nevertheless, the effects of *trans*-18:1 FA consumption are still controversial, because recent animal studies demonstrated neutral or even beneficial effects on cardiovascular disease risk for *trans*-11-18:1, the main *trans* isomer of 18:1 present in milk fat (Wang et al. 2012).

All these aspects underline the interest of modulating milk fat composition by ruminant diet. Supplementation of ruminant diet with plant oils rich in polyunsaturated FA (PUFA) is an efficient way to modify milk FA profile. Both 18:2 n-6 and 18:3 n-3 intakes decreased SFA and increased *trans*-11-18:1 and *cis*-9,*trans*-11-CLA concentrations in milk fat. Besides these major effects, 18:2 n-6 intake increased 18:2 n-6 : 18:3 n-3 ratio, and *trans* isomers of 18:1 (6,7,8, 10, and 12), whereas 18:3 n-3 intake decreased 18:2 n-6 : 18:3 n-3 ratio, and increased *cis*-15-18:1, and *trans* isomers of 18:1 (13–16) (Chilliard et al. 2007). There is also evidence that plant oil supplementation of cow diets decreases milk cholesterol and increases vitamin A concentrations (Nalecz-Tarwacka et al. 2008). Previous experiments have studied the effects on milk FA profile of goats fed vegetable oils rich in either 18:2 n-6 (e.g. sunflower or soybean oils) or 18:3 n-3 (e.g. linseed or rapeseed oils) (Bouattour et al. 2008; Bernard et al. 2009b; Martinez Marin et al. 2012). However, no previous study has used hemp seed oil (HSO) rich in 18:2 n-6 and 18:3 n-3 (59.8 and 18.2% of total FA, respectively) (Da Porto et al. 2012).

Nevertheless, dietary lipid supplementation could also have negative effects in terms of animal health status such as the liver function. The liver is an important site of metabolism of long-chain FA. Dietary PUFA supplementation can lead to increased non-esterified FA (NEFA) concentrations in blood and increased hepatic uptake of these NEFA. The NEFA taken up by the liver may be esterified to form triglycerides, which can accumulate in the liver and cause hepatic steatosis (Grum et al. 1996). Furthermore, little is known about the effect of dietary lipid supplementation on the plasma lipid profile in dairy ruminants, and especially in goats (Bernard et al. 2009a; Li et al. 2012).

Accordingly, the aims of the present study were to evaluate the effect of a diet supplemented with HSO on the lipid metabolism, through the plasma lipid profile, liver function, concentrations of FA, cholesterol, and vitamin A in goat milk. We tested the hypothesis that the HSO supplementation could result in a healthier milk fat composition in dairy goats (with decrease in SFA and cholesterol concentrations, and increase in *cis*-9,*trans*-11-CLA and vitamin A concentrations), implicating changes in rumen biohydrogenation of PUFA and mammary lipogenesis (Chilliard et al. 2007). The subsequent hypothesis is that HSO could affect the plasma lipid profile and liver function by increasing the level of circulating lipids, and activities of some hepatic enzymes as alanine aminotransferase (ALAT) and γ-glutamyltransferase (γ-GT) (Rafalowski and Park 1982; Grum et al. 1996).

MATERIAL AND METHODS

Goats and diets. The experiment was conducted on 10 Carpathian goats in midlactation (at 13 ± 1 week of lactation, average body weight 37.4 ± 3.5 kg at the beginning of the experiment) within July 5–August 4, 2011 on the premises of the Laboratory Animal Facility building of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca (Romania). From May 1 until the beginning of the experiment, the goats grazed on grassland pasture. The goats were then selected and divided into two groups based on their body weight, milk yield, and milk fat content, and received one of the two diets for the whole 31-day experimental period. The feed distribution was collective, per group. The animals were gradually adapted to the experimental diets (4-day adjustment period with distribution of increasing amounts of oil). The control group (C) received daily a basal diet consisting of 60% alfalfa hay and 40% concentrate. The experimental group (HSO) received the same diet in which a part of the concentrate was replaced

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Table 1. Ingredient and fatty acid composition of experimental diets

	Duration of supplementation			
	day –1		day 29	
	C[a]	HSO[a]	C[a]	HSO[a]
Ingredients (% of DM)				
Alfalfa hay	53.68	53.89	56.59	63.03
Concentrate[b]	46.32	46.11	43.41	25.46
Soybean meal	0.00	0.00	0.00	6.72
Hemp seed oil[c]	0.00	0.00	0.00	4.79
Diet fatty acid composition (g/kg DM)				
14:0	0.09	0.09	0.11	0.21
16:0	4.80	4.80	5.28	23.30
18:0	0.86	0.86	0.99	6.78
cis-9-18:1	2.83	2.82	4.03	22.81
18:2 n-6	7.19	7.17	8.97	106.56
18:3 n-3	1.62	1.62	2.34	37.40

C = control diet, HSO = diet supplemented with hemp seed oil, DM = dry matter

[a]values of ether extract are calculated according to Sauvant et al. (2004) and are equal to 2.9 and 2.9% (on DM basis) for C and HSO diets on day –1, and 2.9 and 7.5% for C and HSO diets on day 29, respectively

[b]concentrate composition (g/100 g): wheat (21), corn (40), sunflower meal (20), soybean meal (3), wheat bran (7.5), alfalfa flour (5), premix (3.5). Premix contained minerals (%): Ca (24.25), P (7.62), Mg (0.30), Na (5.22); trace elements (mg/kg): Zn (2064), Cu (275.20), Fe (1720), Mn (2064), I (25.80), Se (3.44), Co (3.44); vitamins: A (400 000 UI/kg), D_3 (40 000 UI/kg), E (1200 mg/kg); antioxidants (3900 mg/kg)

[c]hemp seed oil contained (g/100 g of total FA): 14:0 (0.03), 16:0 (6.54), 18:0 (2.73), *cis*-9-18:1 (10.91), 18:2 n-6 (55.78), 18:3 n-3 (20.65)

by 93 g/day (4.7% of dry matter (DM) intake) of HSO (Canah International, Salonta, Romania). An extra amount of soybean meal was incorporated into the concentrate of the experimental diet to balance nitrogen intake between the two diets. The ingredient composition and FA composition of diets are presented in Table 1. The diets were formulated so as to cover 100–110% of the energy and protein requirements of the animals (INRA 2007).

The goats were milked manually twice daily at 8:00 and 16:00 h. The two groups were housed in the same stall, were separated by a metal grille, had free access to water, and were fed two equal meals after each milking. The feedstuffs distributed and refusals were recorded 24 h before the start of lipid supplementation (day –1) and then on days 1, 8, 15, 22, and 29 throughout the experiment. Body weight of goats was recorded at the beginning and end of the experimental period, after the milking and before the morning meal. All the experimental procedures were approved by the Committee of Bioethics in Scientific Research of the Iuliu Haţieganu University of Medicine and Pharmacy Cluj-Napoca (Romania) and the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca (Romania), and were in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Sampling, measurements, and analyses. Blood samples were collected from the jugular vein on days 1 and 31, before the meal and weighing and after the morning milking, into tubes containing lithium heparin (17 IU/ml). Hematocrit was determined after centrifugation at 12 000 rpm for 3 min. Plasma was obtained by centrifugation at 3000 rpm for 5 min, and then stored at –20°C until chemical analyses.

The plasma concentrations of cholesterol, triglycerides, phospholipids, and total lipids were determined by a visible (Vis) colorimetric method using specific reagent kits (cholesterol kit 4001210L, triglycerides kit 4001415L, phospholipids kit 40014008, total lipids kit 4001410L; all Hospitex Diagnostics, Florence, Italy). Plasma activities of ALAT and γ-GT were determined by an ultraviolet (UV)

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kinetic method using specific reagent kits (ALAT kit 4001755L, γ-GT kit 4001703L; both Hospitex Diagnostics). Analyses were performed using an UV-VIS Screen Master Touch spectrophotometer (Hospitex Diagnostics).

Samples of alfalfa hay (100 g), concentrate (100 g), soybean meal (100 g), and feed refusals (10 g) were collected 24 h before the start of lipid supplementation and on days 8, 15, 22, and 29 of the experimental period, and stored at −20°C. HSO was sampled on days 8, 15, 22, and 29 of the experimental period. The DM of ingredients was determined after drying at 105°C for 24 h. Samples of feeds (except HSO) were lyophilized using Thermovac TM-20 (Froilabo, Ozoir-La-Ferrière, France). The FA composition was determined on ground lyophilized samples of feeds after total lipid extraction and methylation, as described by Sukhija and Palmquist (1988). Tricosanoate (Sigma-Aldrich, Saint-Quentin Fallavier, France) was used as internal standard. The FA from HSO were directly transesterified. HSO was analyzed also for total carotenoids, according to Britton et al. (2004) with some modifications. Briefly, 12 ml HSO were dissolved in 10 ml diethyl ether and then saponified with 22 ml 30% KOH under continuous stirring for 24 h in darkness conditions. Following saponification, the sample was extracted using diethyl ether and then washed successively with a solution of 0.85% NaCl. The remaining water from the ether phase containing the carotenoids was removed using anhydrous sodium sulphate. The solution of carotenoids in diethyl ether was used as such for the determination of total carotenoids content by an UV-Vis spectrophotometer Jenway 6315 (Jenway, Dunmow, UK). The results were calculated using the absorption values at 443 nm according to the formula:

$$X = (A \times V)/(2500 \times 100)$$

where:

X = weight of carotenoids in the sample (mg)

A = absorbance (λ_{max} = 443 nm)

V = sample volume (ml)

2500 = $A^{1\%}_{1\ cm}$ (specific absorbance of coloured carotenoids)

Milk yield of individual goats was recorded (from two consecutive milkings) 24 h before the start of lipid supplementation (day −1) and on days 8, 15, 22, and 29 during the experiment. Samples of milk (80 ml) were collected from each goat over two consecutive milkings, 24 h before the start of lipid supplementation (day −1) and then on days 8, 15, 22, and 29 of the experimental period. Each sample of individual milk was obtained by pooling 60% of the morning milk with 40% of the evening milk, based on the morning and evening milk yield. The highest morning milking percentages recorded for all animals were 71.97 and 73.36% in the C and HSO groups, respectively, whereas the lowest morning milking percentages recorded for all animals were 50.37 and 59.60% in the C and HSO groups, respectively. The morning milking percentage was calculated according to the formula:

(morning milk yield (kg)/total daily milk yield (kg)) × 100.

The daily composite sample for each goat was divided into two sets of milk sub-samples. The first set of sub-samples (40 ml) was stored at 4°C until determination of milk fat and protein contents, using an automated Ekomilk Ultrasonic Milk Analyzer (EON Trading LLC, Delaware, USA) that analyzes milk composition by ultrasonic technology according to Venturoso et al. (2007). A second set of milk sub-samples (40 ml) was lyophilized using Floor Model Freeze Dryer (ilShin America, Hialeah, USA) and stored at −80°C until chemical analyses.

Milk FA composition was determined as described by Ferlay et al. (2010) with some modifications. The FA methyl esters (FAME) were prepared by direct *trans* methylation. Briefly, 2 ml sodium methanolate (0.5 mol/l) and 1 ml hexane were mixed with the lyophilized milk at 50°C for 15 min, followed by the addition of 1 ml 12 N 5% HCl in methanol (v/v) at 50°C for 15 min. The FAME were washed with a saturated K_2CO_3 solution and recovered with 1.5 ml hexane. The FAME were injected (0.6 µl) by autosampler into an Agilent 7890A gas chromatograph equipped with a flame ionisation detector (Agilent Technologies, Wilmington, USA). The FAME from all the samples were separated on a 100 m × 0.25 mm i.d. fused-silica capillary column (CP-Sil 88; Chrompack, Middelburg, the Netherlands). The injector temperature was maintained at 255°C and the detector temperature at 260°C. The initial oven temperature was held at 70°C for 1 min, increased by 5°C/min to 100°C (held for 2 min), and then increased by 10°C/min to 175°C (held for 42 min), and 5°C/min to a final temperature of 225°C (held for 15 min). The carrier gas was hydrogen, and constant pressure (158.6 kPa) was maintained during analysis. A reference standard butter (CRM 164; Commission

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of the European Communities, Community Bureau of Reference, Brussels, Belgium) was used to estimate correction factors for short-chain FA (C4:0 to C10:0). Identification of FAME was accomplished by comparison to a standard mixture (Nu-Chek-Prep, Inc., Elysian, USA). Mixtures of *cis*/*trans* (9–12) isomers of linoleic acid methyl ester and *cis* and *trans* (9–11) and (10–12) isomers of CLA methyl esters (Sigma-Aldrich) were used to complete the identification. Furthermore, the yields of main individual FA in milk fat (see Supplementary Table S3) were calculated according to Glasser et al. (2007) as follows:

FA yield = fat yield (g) × 0.933 ×
× FA concentration (g/100 g of total FA)

The cholesterol concentration of lyophilized milk was determined in total lipid extracts according to the method of Folch et al. (1957) with some modifications. Milk lipid fraction containing cholesterol was extracted with 20 ml methanol and 40 ml chloroform. The mixture was agitated for 15 min and then washed in a separating funnel. The lower chloroform phase was dried over anhydrous Na_2SO_4, evaporated to dryness, and recovered in 1 ml of chloroform. Cholesterol was derivatized with 480 µl of a mixture of bistrimethylsilyl-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (2 : 1) for 2 h at 60°C and then quantified by gas chromatography. Derivatized standards and samples (2 µl) were injected into a Shimadzu GC 2010 gas chromatograph equipped with a flame ionization detector (Shimadzu, Kyoto, Japan) and separated with a fused silica capillary column CP-Sil 5CB (Varian Inc., Palo Alto, USA) 25 m × 0.25 mm, film thickness 0.25 µm. Injector temperature was set at 260°C, and detector temperature at 290°C. The initial oven temperature was held at 130°C for 3 min, increased by 10°C/min to 200°C (held for 3 min), and then by 20°C/min to a final temperature of 290°C (held for 5 min). The carrier gas was helium, with a flow rate of 1.9 ml/s. The internal standard (Sigma-Aldrich) and cholesterol peaks were determined by area measurement. Cholesterol concentration was expressed in mg/100 g fluid milk, from the amount of lyophilized milk weighed from a known amount of fluid milk.

Milk vitamin A concentration of lyophilized milk was determined by HPLC using the method described by Andrei et al. (2009) with some modifications. Vitamin A in hexanoic phase was injected into an HPLC system equipped with pumps (Shimadzu LC-20 AT; Shimadzu, Kyoto, Japan), a photodiode-array detector (SPDM20A Prominence; Shimadzu), and a reversed-phase chromatographic column (Supelco Discovery RP18, Nucleosil 5 µm, 250 × 4.6 mm; Sigma-Aldrich) with a mobile phase of acetonitrile : methanol (85 : 15, v/v) in an isocratic system. Quantification was obtained from calibration curves using a standard solution of *trans*-total retinol (Sigma-Aldrich). Vitamin A concentration was expressed in µg/ml fluid milk, from the amount of lyophilized milk weighed from a known amount of fluid milk.

Statistical analyses. Data for plasma lipid profile, ALAT and g-GT activities obtained on day 1 were used as covariates. Data recorded on day 31 were analyzed using the MIXED procedure of SAS (Statistical Analysis System, Version 9.1, 2003),

Table 2. Effect of dietary supplementation of hemp seed oil on plasma lipid profile and enzymatic activities in dairy goats

	Diet		SEM	Significance (*P*-value)
	C	HSO		
Hematocrit (%)	30.70	32.30	1.177	ns
ALAT (IU/l)	15.79	18.72	1.644	ns
γ-GT (IU/l)	31.85	37.80	3.621	ns
Cholesterol (mg/dl)	77.31	93.07	7.380	ns
Triglycerides (mg/dl)	59.87	56.92	2.011	ns
Phospholipids (mg/dl)	95.19	97.33	11.762	ns
Total lipids (mg/dl)	287.41	347.64	16.940	*

C = control diet, HSO = diet supplemented with hemp seed oil, ALAT = alanine aminotransferase, γ-GT = gamma-glutamyltransferase, IU = international unit, SEM = Standard Error of the Mean, ns = not significant
ns ($P > 0.10$), *$P < 0.05$

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with diet and covariate as fixed effects, and goat as random effect.

Data for milk yield and composition, milk FA, cholesterol, and vitamin A concentrations recorded the day before the addition of oil (day –1) were used as covariates. These data were analyzed as repeated measures using the MIXED procedure of SAS. The statistical model included: day, diet, goat, diet × day interaction, covariate, and residual error. Fixed effects included covariate, day, diet, and interaction. Goat was the random effect.

RESULTS

Plasma lipid profile and enzymatic activities. The HSO supplementation only increased plasma total lipid concentration ($P < 0.05$). The activities of γ-GT and ALAT, and the concentrations of hematocrit, cholesterol, triglycerides, and phospholipids did not differ between the diets (Table 2).

Dairy performance, milk fatty acid, cholesterol, and vitamin A concentrations. The HSO supplementation increased milk fat yield (+ 13.21 g/day; $P < 0.001$) and milk fat content (+ 0.89%; $P < 0.001$) (Table 3). Milk fat content changed across the experimental period in both diets ($P < 0.05$), in particular with a decrease on day 15. The HSO supplementation increased milk protein content until day 15, and then decreased it until day 29 (diet × time interaction, $P < 0.05$).

The HSO supplementation markedly changed milk FA composition compared with C diet (Table 4; Supplementary Tables S1 and S2). The HSO supplementation decreased ($P < 0.001$) the milk fat concentration of 20:4 n-6, but increased ($P < 0.05$) the milk fat concentrations of *trans*-4-18:1, *trans*-9,*trans*-12- and *cis*-9,*trans*-12-18:2.

The milk fat concentrations of 6:0, 24:0, *trans*-9-14:1, *trans*-6-16:1, *cis*-9-, *cis*-13- and *cis*-15-18:1, 18:3 n-6, 20:4 n-6, and 22:6 n-3 changed slightly across the experimental period in both diets.

The responses of concentrations of most FA to HSO supplementation were transient according to time (diet × time interaction), and the kinetics of the responses were different according to the FA considered (Table 4, Supplementary Tables S1 and S2). Milk fat concentrations of total SFA ($P < 0.001$), 12:0 ($P < 0.01$), 14:0 ($P < 0.001$), *cis*-9-14:1 ($P < 0.001$), anteiso 17:0 ($P < 0.001$), and *cis*-9-18:1/18:0 desaturase ratio ($P < 0.001$) declined with HSO diet (compared with C diet), except on

Table 3. Effect of dietary supplementation of hemp seed oil on dry matter intake, milk yield, composition, cholesterol, and vitamin A concentrations in dairy goats

	Diet		SEM	Duration of supplementation								Significance (P-value)			
				day 8		day 15		day 22		day 29					
	C	HSO		C	HSO	C	HSO	C	HSO	C	HSO		diet	time	diet × time
Total DM intake (kg/day per group of 5 animals)	8.57	8.61		9.24	9.40	9.39	9.40	9.78	9.82	10.16	9.82				
Milk yield (l/day)	1.28	1.33	0.041	1.32	1.31	1.19	1.31	1.30	1.34	1.30	1.35	0.051	ns	*	ns
Fat content (%)	2.70	3.59	0.120	2.65	3.84	2.55	3.09	3.06	3.79	2.55	3.65	0.215	***	*	ns
Protein content (%)	3.16	3.28	0.034	3.09	3.45	3.12	3.26	3.25	3.24	3.18	3.17	0.057	*	ns	*
Fat yield (g/day)	34.53	47.74	1.716	34.93	50.65	30.23	40.59	39.81	50.75	33.13	48.99	3.012	***	*	ns
Protein yield (g/day)	40.37	43.60	1.245	40.78	45.24	37.18	42.84	42.24	43.52	41.26	42.80	1.619	+	*	ns
Cholesterol (mg/100 g)	14.63	11.83	1.333	15.83	16.96	13.17	9.85	15.60	10.54	13.92	9.99	2.162	ns	+	ns
Vitamin A (μg/ml)	0.167	0.151	0.023	0.135	0.126	0.141	0.125	0.199	0.162	0.194	0.190	0.027	ns	**	ns

C = control diet, HSO = diet supplemented with hemp seed oil, DM = dry matter, SEM = Standard Error of the Mean, ns = not significant
ns ($P > 0.10$), + ($P < 0.10$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

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Table 4. Effect of dietary supplementation of hemp seed oil on main milk fatty acid concentrations in dairy goats

	Diet		SEM	Duration of supplementation								SEM	Significance (*P*-value)		
				day 8		day 15		day 22		day 29					
	C	HSO		C	HSO	C	HSO	C	HSO	C	HSO		diet	time	diet × time
Fatty acids (g/100 g of total fatty acids)															
4:0	2.40	2.46	0.065	2.25	2.28	1.98	2.37	2.69	2.72	2.69	2.49	0.090	ns	***	**
6:0	2.67	2.69	0.071	2.66	2.52	2.34	2.61	2.90	2.87	2.80	2.76	0.141	ns	*	ns
8:0	2.95	2.87	0.098	3.08	2.72	2.69	2.82	3.11	2.99	2.91	2.94	0.192	ns	ns	ns
10:0	9.64	8.44	0.373	11.53	7.84	9.76	8.55	8.44	8.59	8.83	8.79	0.708	*	ns	*
12:0	4.34	3.15	0.225	5.82	2.94	5.09	3.41	2.98	3.13	3.47	3.13	0.377	**	**	**
14:0	9.31	7.56	0.377	11.10	6.16	10.41	7.57	6.90	8.07	8.83	8.46	0.579	**	*	***
16:0	24.20	20.74	0.671	26.08	17.44	25.32	18.34	19.74	23.64	25.66	23.55	1.063	**	*	***
18:0	9.70	11.59	0.740	5.34	9.95	6.44	9.83	15.11	13.14	11.93	13.44	1.064	+	***	**
cis-9-18:1	17.43	15.35	1.174	13.92	14.83	17.72	12.26	20.89	17.66	17.20	16.65	1.837	ns	*	ns
cis-9,*trans*-13-18:2	0.27	0.36	0.017	0.26	0.43	0.27	0.43	0.30	0.27	0.23	0.32	0.029	***	*	**
trans-11,*cis*-15-18:2	0.12	0.30	0.029	0.10	0.48	0.06	0.33	0.20	0.19	0.15	0.21	0.043	***	*	***
cis-9,*trans*-11-CLA	0.49	2.14	0.174	0.51	3.63	0.47	3.72	0.57	0.65	0.40	0.56	0.232	***	***	***
18:2 n-6	2.40	2.77	0.138	2.78	4.39	2.95	2.97	1.89	1.90	1.96	1.83	0.228	+	***	**
18:3 n-3	0.94	1.21	0.159	0.78	1.34	0.85	0.89	1.15	1.14	0.99	1.48	0.226	ns	ns	ns
20:0	0.26	0.34	0.020	0.21	0.32	0.21	0.38	0.31	0.30	0.29	0.37	0.028	**	ns	**
22:0	0.12	0.13	0.011	0.13	0.11	0.11	0.12	0.13	0.12	0.11	0.16	0.016	ns	ns	+
24:0	0.06	0.06	0.009	0.05	0.04	0.05	0.05	0.07	0.07	0.06	0.10	0.012	ns	*	+
Total SFA	70.97	64.59	0.762	74.21	56.55	70.51	60.51	66.93	70.27	72.23	71.03	1.414	***	***	***
Total OBCFA[a]	5.36	4.58	0.134	6.01	4.26	6.14	4.50	4.60	4.67	4.69	4.89	0.214	***	**	***
Total MUFA	22.77	26.53	0.745	19.15	31.01	22.61	29.03	26.99	23.75	22.31	22.35	1.357	**	+	***
Total *cis*-18:1	18.49	16.63	1.175	15.09	16.60	18.82	13.70	21.89	18.61	18.17	17.60	1.851	ns	+	ns
Total *trans*-18:1	3.06	8.95	0.724	2.67	13.51	2.30	14.51	4.08	4.09	3.20	3.66	0.999	***	***	***
Total *trans* FA	6.63	14.85	0.925	6.61	22.68	6.43	22.16	7.29	7.54	6.18	7.02	1.265	***	***	***
Total PUFA	5.30	7.69	0.287	5.67	11.21	5.87	9.27	5.13	4.95	4.55	5.33	0.464	***	***	***
Total n-6	2.57	2.94	0.131	3.01	4.57	3.15	3.17	2.03	2.05	2.09	1.99	0.231	+	***	**
Total n-3	1.35	1.57	0.185	1.27	1.70	1.35	1.24	1.52	1.45	1.25	1.89	0.270	ns	ns	ns
Desaturase ratio															
cis-9-14:1/14:0	0.02	0.01	0.001	0.02	0.01	0.02	0.01	0.009	0.01	0.01	0.008	0.001	*	***	**
cis-9-18:1/18:0	2.13	1.37	0.177	2.78	1.42	2.88	1.29	1.37	1.44	1.49	1.32	0.220	**	***	***
cis-9,*trans*-11-CLA/ *trans*-11-18:1	0.51	0.42	0.037	0.66	0.44	0.74	0.41	0.30	0.41	0.36	0.40	0.054	ns	***	***

C = control diet, HSO = diet supplemented with hemp seed oil, CLA = conjugated linoleic acids, SFA = saturated fatty acids (FA), OBCFA = odd- and branched-chain FA, MUFA = monounsaturated FA, PUFA = polyunsaturated FA, SEM = Standard Error of the Mean, ns = not significant

[a]sum of iso 13:0, anteiso 13:0, iso 14:0, iso 15:0, anteiso 15:0, iso 16:0, iso 17:0, anteiso 17:0, and iso 18:0

ns ($P > 0.10$), + ($P < 0.10$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

day 22, when the above FA increased. By contrast, milk fat concentrations of total monosaturated FA (MUFA) ($P < 0.001$), total PUFA ($P < 0.001$), iso 17:0 ($P < 0.01$), 20:0 ($P < 0.01$), *trans*-11-18:1 ($P < 0.001$), *trans*-16-18:1 ($P < 0.01$), *cis*-9,*trans*-13- ($P < 0.01$), and *trans*-11,*cis*-15-18:2 ($P < 0.001$) were increased with HSO supplementation, except on day 22, when these FA concentrations decreased.

Milk fat concentrations of total odd- and branched-chain FA (OBCFA) ($P < 0.001$), 15:0 ($P < 0.001$), 23:0 ($P < 0.05$), and anteiso 15:0 ($P < 0.01$) were decreased by HSO (compared with C diet) from days 8 to 15, but they were then increased from day 22 until day 29. From days 8 to 22 of the experiment, HSO decreased the milk fat concentrations of 17:0 ($P < 0.001$), *cis*-11- ($P <$

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0.05) and *trans*-11-16:1 ($P < 0.01$), and *cis*-9-17:1 ($P < 0.001$), whereas on day 29 the concentrations of these FA were increased with HSO supplementation. Also, the decrease in 11:0 ($P < 0.01$), 13:0 ($P < 0.001$), *cis*-9-10:1 ($P < 0.001$), *cis*-9-12:1 ($P < 0.001$), anteiso 13:0 ($P < 0.001$), and iso 14:0 ($P < 0.05$) milk fat concentrations with HSO supplementation was transient, so that concentrations of these FA were comparable to those from C diet towards the end of the experiment.

HSO supplementation increased the milk fat concentrations of *cis*-12- ($P < 0.001$) and *trans*-5-18:1 ($P < 0.01$) until day 15 and then decreased these FA concentrations. From day 8 to day 22, HSO (compared with C diet) increased total n-6 ($P < 0.01$), 4:0 ($P < 0.01$), *trans*-6,7,8- ($P < 0.001$), *trans*-9- ($P < 0.001$), and *trans*-10-18:1 ($P < 0.05$), and 18:2 n-6 ($P < 0.01$) concentrations in milk fat, whereas on day 29 they were decreased. Supplementation with HSO continuously increased milk fat concentrations of total *trans*-18:1 ($P < 0.001$), total *trans* FA ($P < 0.001$), *trans*-12- ($P < 0.001$) and *trans*-13-18:1 ($P < 0.01$), and *cis*-9,*trans*-11-CLA ($P < 0.001$).

Milk cholesterol and vitamin A concentrations were not affected by HSO supplementation, but milk vitamin A concentration increased from day 22 ($P < 0.05$) in both diets (Table 3).

Milk fatty acid yields. The HSO supplementation markedly influenced milk individual FA yields when compared with C diet (Supplementary Table S3). The HSO supplementation increased the milk fat yield of 4:0 ($P < 0.001$), 6:0 ($P < 0.001$), 8:0 ($P < 0.01$), and 10:0 ($P < 0.05$). The milk fat yield of 12:0 ($P < 0.05$) and 14:0 ($P < 0.01$) was decreased by HSO (compared with C diet) from days 8 to 15, but it was then increased from day 22 until day 29.

DISCUSSION

Plasma lipid profile and enzymatic activities. In the present study, HSO supplementation had no influence on the plasma concentration of triglycerides or phospholipids. The lack of HSO effect on plasma triglycerides or phospholipids is surprising, because lipid supplementation generally increased these plasma lipid fractions (Bernard et al. 2009a; Li et al. 2012). Nevertheless, HSO supplementation increased plasma total lipid concentration in the present study. This increase might be due to the higher lipid intake with the supplemented diet. Our results are in agreement with Rafalowski and Park (1982), who showed that dairy cows receiving increasing amounts of whole sunflower seeds (10–30% of the concentrate) had higher blood total lipid concentration than control cows.

Liver function was evaluated by the activities of γ-GT and ALAT. The γ-GT is a membrane-bound enzyme, found in tissues with high rates of secretion or absorption, among the most notable being the liver (Djuricic et al. 2011). The γ-GT activity increases as a result of damage to the cellular structures of hepatocytes (Lubojacka et al. 2005). The ALAT is a transaminase enzyme present in high concentrations in the cytoplasm of hepatocytes (Kupczynski and Chudoba-Drozdowska 2002). The ALAT is considered one of the most sensitive enzymes in the diagnosis of hepatocellular injury (Tibbo et al. 2008). In our study, the values for the plasma concentrations of γ-GT and ALAT were close to the value ranges (20–50 IU/l for γ-GT and 15.3–52.3 IU/l for ALAT, respectively) of Tibbo et al. (2008) and Djuricic et al. (2011).

In the present experiment, the plasma γ-GT activity was not influenced by HSO supplementation, suggesting a lack of negative effect of lipid supplementation on liver function. Similarly, Nudda et al. (2013) reported no influence on plasma γ-GT when the goats were fed a ryegrass hay-based diet supplemented with 10.5% extruded linseed. In disagreement with our results, Bianchi et al. (2014) reported evidence of an increase in γ-GT activity when dairy ewes were fed silage-based diets supplemented with 6.0% palm oil for 120 days. This negative effect could be due to the nature of this lipid supplement rich in SFA. A lack of negative effect of HSO supplementation on liver function was also confirmed by the absence of alteration of ALAT activity in plasma of goats fed the HSO diet. In contrast to our results, Nudda et al. (2013) reported an increase in ALAT activity when goats were fed a ryegrass hay-based diet supplemented with 10.5% extruded linseed and these authors suggested that an increase in ALAT concentration could be related to a higher milk secretion and, consequently, a higher liver turnover metabolic rate in goats.

Dairy performance. In our study, milk yield was not affected by HSO supplementation, in agreement with Chilliard et al. (2003), Chilliard and Ferlay (2004), Bernard et al. (2009b) with grass hay diets supplemented with 5.8% sunflower or linseed oil, and Bouattour et al. (2008) with diets

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based on dehydrated fescue supplemented with 2.5% soybean oil. The increase in milk fat content due to HSO supplementation is in agreement with Bouattour et al. (2008), who reported an increase in milk fat content (+ 0.67%) with a 2.5% soybean oil supplementation. Similarly, Li et al. (2012) reported that supplementation of an alfalfa hay diet with 5% safflower oil or linseed oil enhanced milk fat content (+ 0.45 and 0.62%, respectively). In fact, recent *in vitro* data indicated that mammary lipogenesis in goats is not affected, and may even be stimulated by intermediates of ruminal biohydrogenation (RBH) of PUFA (Bernard et al. 2013).

HSO supplementation slightly enhanced milk protein content in agreement with Bernard et al. (2009b), who found that supplementation with 5.8% linseed oil of a based-grass hay diet increased milk protein content (+ 0.08%). This increase could be due to a nutritional status less limiting for milk protein synthesis in the HSO group when compared to C group, because milk protein content is essentially influenced by energy balance (Coulon and Remond 1991). Different mechanisms explaining the relationship between energy balance and milk protein content have been proposed. Increased availability of propionate and microbial protein could influence the nutritional physiology of the lactating ruminants and enhance milk protein secretion (Jenkins and McGuire 2006). Furthermore, in our study, an increased microbial protein availability in the intestine of goats fed the HSO diet could be due to the defaunation effect of PUFA provided in high amounts by HSO on rumen protozoa (Nudda et al. 2013).

Milk fatty acid concentrations. The HSO supplementation increased the milk fat concentration of 4:0, with no effect on 6:0 or 8:0 concentrations. An increase in milk fat 4:0 concentration has also been reported by Bernard et al. (2009b) with 5.8% sunflower oil or linseed oil supplementation. However, Bouattour et al. (2008) observed no significant changes in the milk fat 4:0 to 8:0 concentrations from goats fed diets supplemented with 2.5% soybean oil. This result is in line with Chilliard and Ferlay (2004) who reported no changes in 4:0 to 8:0 concentrations after a supplementation of alfalfa hay-based diets with vegetable oils rich in 18:2 n-6 or 18:3 n-3 (5–6% of total DM intake). The HSO supplementation decreased the milk fat concentrations of saturated *de novo* synthesized FA (10:0, 12:0, 14:0, and 16:0), in agreement with previous studies in dairy goats (Bouattour et al. 2008; Bernard et al. 2009b). The decrease in milk fat concentration of 10:0 to 16:0 without changes in 6:0 and 8:0 concentrations could be due to the inhibitory effect of long-chain FA uptake by the mammary gland on the ratio of acetyl-CoA carboxylase to FA synthetase activities (Martinez Marin et al. 2012). According to this last study, Bernard et al. (2009a) reported a decrease in the ratio of acetyl-CoA carboxylase to FA synthetase activities with no reduction in concentrations of 4:0 to 8:0 in milk fat of goats fed diets supplemented with 6% of sunflower or linseed oil.

The HSO supplementation reduced the milk fat concentrations of most OBCFA, in agreement with Bernard et al. (2009b), who reported a decrease in milk fat OBCFA concentration by 5.8% sunflower oil or linseed oil supplementation. The OBCFA are largely synthesized by rumen bacteria, so that reductions in milk concentrations of these FA could be due to an inhibitory effect of dietary PUFA on rumen microbial activity (Vlaeminck et al. 2006).

The lack of *cis*-9-18:1 response to HSO supplementation in our study is in agreement with Chilliard et al. (2003), Chilliard and Ferlay (2004), Bernard et al. (2009b), although some studies have shown increases in milk fat *cis*-9-18:1 concentration by vegetable oil supplementation (Bouattour et al. 2008; Li et al. 2012). Also, we reported a decrease in *cis*-9-14:1/14:0 ratio, the best indicator of Δ-9 desaturase activity (Arnould and Soyeurt 2009), and in *cis*-9-18:1/18:0 and *cis*-9-*trans*-11-CLA/*trans*-11-18:1 ratios with HSO supplementation. Our results suggest that the mammary desaturation capacity could be limited with the increased supply of 18:0 to the mammary gland by the supplemented diet, which could partly explain the lack of variation in *cis*-9-18:1 milk fat concentration with HSO supplementation. Another possible explanation could be that the desaturation activity is inhibited by the intermediates of RBH of dietary PUFA (Chilliard et al. 2007).

HSO supplementation significantly increased the milk fat concentration of *trans*-18:1 isomers, and notably *trans*-11-18:1 (+ 3.87 g/100 g of total FA), in agreement with Chilliard et al. (2003) and Chilliard and Ferlay (2004). This increase could be due to the high intakes of 18:2 n-6 and 18:3 n-3 with the HSO diet, because both these FA have *trans*-11-18:1 as main intermediate in their RBH pathways (Chilliard et al. 2007).

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HSO supplementation has been shown to be an effective way to increase the milk fat concentration of *cis*-9,*trans*-11-CLA (+ 1.39 g/100 g of total FA) in the present study. There are few studies in dairy goats on the kinetics of responses of milk FA concentrations to vegetable oils. The response of goat milk *cis*-9,*trans*-11-CLA concentration to vegetable oils (linseed oil, oleic sunflower oil) has been shown to peak two weeks after the beginning of supplementation, and to persist for at least ten weeks of lipid supplementation (Chilliard et al. 2007). Similarly, in dairy cows, supplementing a grass-hay diet with 5% linseed oil for 20 days gradually enhanced milk fat *cis*-9,*trans*-11 CLA concentrations from the beginning to the end of the experiment (Roy et al. 2006). However, in the present study, the responses of *cis*-9,*trans*-11-CLA milk fat concentrations to HSO supplementation were transient, with values peaking during the second week after the beginning of supplementation, and decreasing from the third week. These changes could be linked to slight differences in the forage : concentrate ratio during the experiment, and so to an adaptation of the rumen microbial ecosystem to HSO supplementation.

HSO supplementation increased milk fat 18:2 n-6 concentration (+ 0.33 g/100 g of total FA) like in Bouattour et al. (2008) (+ 0.41 g/100 g of total FA) after supplementation with soybean oil (2.5% of total DM intake). However, the relatively low increase in milk fat 18:2 n-6 concentration in our study is consistent with previous studies in goats, indicating that the milk 18:2 n-6 concentrations rarely exceeded control values by more than 1.5% with diets supplemented with seeds or oils rich in 18:2 n-6 such as sunflower or soybean (Chilliard et al. 2007). Also, milk fat 18:3 n-3 concentration was not significantly changed with HSO supplementation, in agreement with Bernard et al. (2009b), who showed no effect on milk fat 18:3 n-3 concentration with 5.8% linseed oil supplementation of grass-hay diet. Although dietary 18:3 n-3 intake amount was greater in the HSO than in the C diet, the lack of increase in milk fat 18:3 n-3 concentration with HSO supplementation could be partially attributed to the fact that most of the dietary 18:3 n-3 from the HSO diet was supplied in the free form (oil) and thus was highly vulnerable to biohydrogenation (Chilliard et al. 2007).

Milk cholesterol and vitamin A concentrations. The values of milk cholesterol concentration in the present study are comparable to those observed by Fletouris et al. (1998) with goat milk obtained from a local milk industry in Greece (14.4 mg/100 g). In our study, HSO supplementation had no influence on milk cholesterol concentration, but it numerically increased plasma cholesterol concentration. Similarly, Rafalowski and Park (1982) reported no effect of increasing amounts of whole sunflower seeds (1.1–3.2% of ether extract in the DM diet) on milk cholesterol concentration from Holstein cows, whereas serum cholesterol concentration was strongly increased. The lack of the effect of lipid supplementation on milk cholesterol concentration could be due to the fact that milk cholesterol is mainly synthesized through *de novo* processes independent of the ruminant diet (Strzalkowska et al. 2010). Vitturo et al. (2009) suggested that only 20% of the total milk cholesterol is derived from mammary *de novo* synthesis, and the remaining cholesterol in milk originates from the uptake of serum cholesterol obtained through hepatic synthesis.

At variance with our results, supplementation of a partial mixed ration with 5.2% soybean oil decreased milk cholesterol concentration (–6.37 mg/100 g between the beginning and end of the experimental period), and increased plasma cholesterol concentration in Holstein cows (Altenhofer et al. 2014). Similarly, addition of 275 g or 550 g of rapeseed oil decreased cow milk cholesterol concentration by 8 and 13%, respectively (Precht 2001). The differences between these studies and our study could be due to a species effect (cow vs goat) or to the nature of the oil supplementation (rich in either 18:2 n-6 or 18:3 n-3).

The values of milk vitamin A concentration in our study are comparable with those reported by Kondyli et al. (2007) with the milk from indigenous Greek goats during the entire lactation (0.13 µg/ml on average). Vitamin A in milk may be derived from forages, concentrates (cereals, oilseeds), and mineral-vitamin supplements in the ruminant diet or it may be formed from dietary β-carotene (Ferlay et al. 2013). The HSO supplementation had no effect on milk vitamin A concentration, suggesting that there was no difference in vitamin A and β-carotene intakes between C and HSO diets, according to the experimental design, although HSO is a source of β-carotene (with a supply of total carotenoids of 0.539 mg/100 ml). Our results are in disagreement with Puppel et al. (2013),

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who showed that supplementation of diet with different lipid sources (fish oil or linseed) for 21 days increased milk vitamin A concentration in all dietary treatments (from 0.170 to 1.333 µg/ml) in Polish Holstein-Friesian cows. Similarly, Nalecz-Tarwacka et al. (2008) reported an increase in milk vitamin A concentration (+ 0.147 µg/ml) with 4% linseed supplementation for 28 days in Holstein-Friesian cows.

CONCLUSION

The present study shows that HSO supplementation increased milk fat and protein contents, without affecting plasma lipid profile or liver function. However, HSO supplementation increased plasma total lipid concentration, suggesting that this lipid supplementation may have a potential hyperlipidemic effect in dairy goats.

HSO supplementation strongly altered milk FA composition, with a decrease in *de novo* synthesized FA (10:0-16:0) and an increase in 4:0, 18:0, *cis*-9,*trans*-11-CLA and PUFA concentrations, suggesting that HSO can be used to increase the milk fat concentrations of FA with a putative positive effect on human health. Nevertheless, HSO supplementation strongly increased milk fat *trans*-18:1 concentrations. The milk responses of several FA to HSO supplementation were transient, and declined during the experimental period. Finally, HSO had no effect on milk cholesterol or vitamin A concentrations.

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