Fatty acid profiles of rumen fluid from sheep fed diets supplemented with various oils and effect on the rumen ciliate population

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ABSTRACT: The profile of trans-fatty acids and the protozoan population were evaluated in four ruminally fistulated sheep fed a diet with meadow hay: barley grain ratio (80:20%) plus sunflower oil (SO), rapeseed oil (RO) or linseed oil (LO) (5% wt/wt). The concentrate was daily mixed with individual oils and offered at 07.00 h. A 4 × 4 Latin square with 4-week periods was used. The concentration of trans-vaccenic acid (TVA) was the highest 4 h after feeding (36.1 g/100 g FA with SO; 34.5 g/100 g FA with RO) and then decreased with the time after feeding ($P < 0.05$). The concentration of *cis9, trans*11 conjugated linoleic acid (c9,t11-CLA) with RO increased from 3.23 g/100 g FA (2 h after feeding) to 4.67 g/100 g FA (4 h after feeding). The concentration of c9,t11-CLA with SO increased from 2.09 g/100 g FA (2 h after feeding) to 2.31 g/100 g FA (4 h after feeding). The concentration of c9,t11-CLA with LO was the highest 4 h after feeding (2.07 g/100 g FA). Overall effects of the oil supplements and time after feeding on short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA) and long-chain fatty acids (LCFA) in the rumen fluid were evident. A strong interaction of oil supplements and time after feeding was detected in the concentration of UFA and SFA ($P < 0.001$). A significant effect of LO on the rumen ciliate population was observed; the total protozoan concentration and the number of *Entodinium* spp. were decreased as well as *Dasytricha ruminantium*, *Isotricha* spp., *Polyplastron multivesiculatum*, *Ophryoscolex c. tricoronatus* and *Eremoplastron dilobum*.

Keywords: sunflower oil; rapeseed oil; linseed oil; fatty acids; rumen ciliates

Ruminant diets contain a relatively small amount of lipids. Animal studies indicated that the supplementation of diet with lipids influences the concentration and types of fatty acids formed in the rumen. Conjugated linoleic acids (CLA) are naturally occurring fatty acids in foods obtained from ruminants. The most representative is *cis9, trans*11 conjugated linoleic acid. It is formed as an intermediate isomer during the rumen biohydrogenation of linoleic acid to trans-vaccenic acid and stearic acid by rumen bacteria (Polan et al., 1964; Harfoot and Hazlewood, 1988) or from the endogenous conversion of trans-vaccenic acid by the enzyme delta-9-desaturase in animal tissues (Griinari et al., 2000; Corl et al., 2001). Recent studies suggest that the protozoa can play an important role in the synthesis of TVA and CLA in the rumen (Devillard et al., 2006; Yánez-Ruiz et al., 2006; Or-Rashid et al., 2007). In addition, it is well known that the rumen protozoa contain proportionally more unsaturated fatty acids than the rumen bacteria (Viviani, 1970; Devillard et al., 2006). Previously, we investigated the effect of sunflower oil and linseed oil on the production of trans fatty acids *in vitro* (Jalč et al., 2005a,b). This experiment was conducted (1) to determine the effect of various oil supplements (sunflower, rapeseed, or linseed) on sheep rumen fatty acid profiles, especially *cis9, trans*11-CLA and trans-vaccenic acid, and (2) to compare the effect of various oil supplements (sunflower, rapeseed, or

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linseed) on the protozoan population in the rumen fluid of sheep.

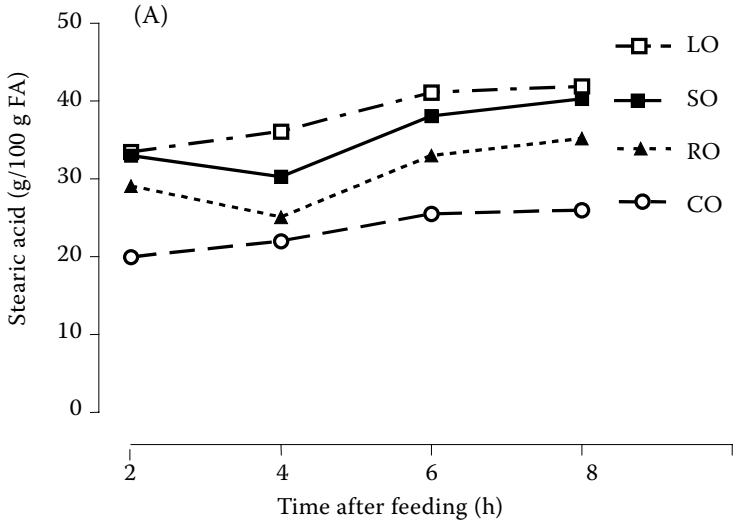
MATERIAL AND METHODS

Four ruminally fistulated sheep (Slovak Merino) were used in a 4 × 4 Latin square design during 4-week periods to evaluate responses to feeding diets. The diets consisted of meadow hay and barley grain (80:20; 1 200 g DM/day) supplemented (60 g/day) with sunflower oil (SO), rapeseed oil (RO) or linseed oil (LO). The oils rich in linoleic acid (SO, 53.3 g/100 g FA), oleic acid (RO, 60.5 g/100 g FA) and α-linolenic acid (LO, 50.4 g/100 g FA) were used (Table 1). One sheep was a control (CO). To allow sheep to adapt to oil-supplemented diets, all the sheep were fed a diet supplemented with an oil mixture made up of sunflower oil, rapeseed oil plus linseed oil (20 g/day +20 g/day +20 g/day, respectively) 4 weeks prior to the first experimental period. Supplemental oils were homogeneously mixed with the concentrate. The experimental animals had different types of ciliate population (A and B; Eadie, 1962, 1967). Two animals had a B-type population (*Epidinium* and *Eudiplodinium* spp.) and two animals had an A-type population (*Polyplastron* and *Ophryoscolex* spp.) which is antagonistic to the B-type population. To obtain the uniform rumen protozoan population, the one-shot interfusion of the rumen fluid from all four sheep with following inoculation via ruminal fistula before experiments was performed. The change in the protozoan population to A-type was observed during the adaptation period. This intervention in the ciliate population of sheep allowed reaching homogeneous samples for the ciliate population analysis. During the next four-week experiments, the concentrate was daily mixed with individual oils and offered at 07.00 h throughout each of the four periods in the replicated 4 × 4 Latin square design. Hay was given twice daily. The sheep were housed separately in pens with a free access to water. Rumen fluid samples were collected 2, 4, 6 and 8 h after feeding (at 09.00, 11.00, 13.00, and 15.00 h) on the seventh day of each experimental period. The samples for ciliate counts were collected 2 h after feeding on the seventh day of each experimental period and the strained rumen fluid was fixed with an equal volume of 8% of formaldehyde. The concentration of the ciliate protozoa was counted microscopically according to the procedure described by Coleman (1978). Protozoa were identified according to Dogiel (1927) and Ogimoto and Imai (1981). The rumen fluid was strained through four layers of cheesecloth. Lipids in the strained rumen fluid were extracted by the method of Bligh and Dyer (1959) with purification of samples with 20% of HCl. Extracted lipids in tubes were dissolved in 1 ml of hexane and 1 ml of internal standard (tridecanoic acid; Fluka, Sigma – Aldrich). 2 ml of transesterification reagent (1N methanolic sodium methoxide; Baše, 1978) were added to the mixture. The mixture was held in a water bath at 50°C for 30 min. After addition of 3 ml of 3N methanolic HCl (Supelco, USA) the mixture was incubated in the water bath at 50°C for 1 h. To separate the hexane layer in the mixture, 1 ml of hexane and 1 ml of distilled water was added. Finally, the mixture was centrifuged at 200 g for 5 min. The hexane layer was used for the determination of fatty acid methyl esters by a gas chromatography. The samples were injected with a split less injector into a Perkin-Elmer Clarus 500 gas chromatograph (Perkin-Elmer, Inc. Shelton, Connecticut, USA). The gas chromatograph was equipped with a capillary column DB-23 (60 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies) and a flame ionization detector (constant flow, hydrogen 40 ml/min, air 400 ml, 260°C). The analyses of fatty acids (0.5 μl methyl esters in hexane injected at a 30:1 split ratio) were carried out under a temperature gradient (130°C for 1 min; 130–170°C at program rate 6.5°C/min; 170–206°C at program rate 1°C/min; 206–240°C

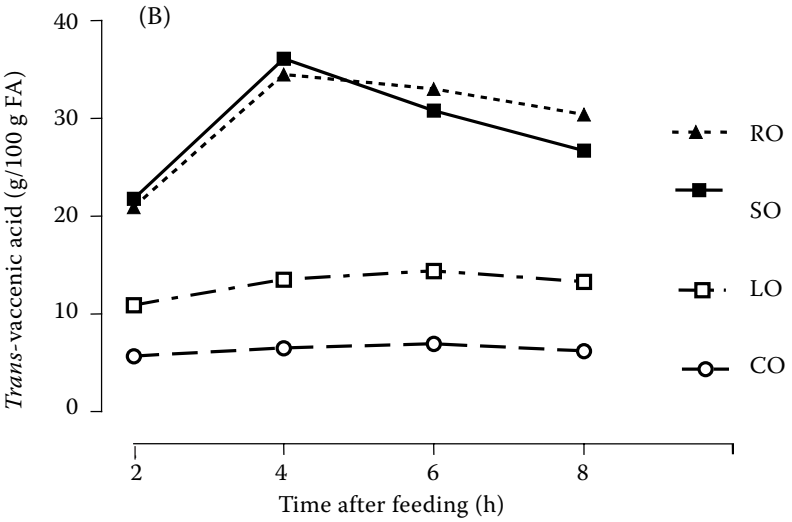
Table 1. Fatty acid compositions of oils (SO, sunflower oil; RO, rapeseed oil; LO, linseed oil; g/100 g FA)

Fatty acid	SO	RO	LO
C16:0 palmitic	5.7	5.3	4.5
C16:1 palmitoleic	–	0.3	0.02
C18:0 stearic	3.3	1.7	3.99
C18:1*n*-9 oleic	33.9	60.5	22.5
C18:2*n*-6 linoleic	53.3	23.0	17.6
C18:3*n*-3 α-linolenic	1.1	8.5	50.4
C20:1*n*-9 eicosenoic	0.3	1.5	–
SFA	9.4	7.0	8.53
MUFA	34.2	62.0	22.8
PUFA	54.3	31.0	68.3

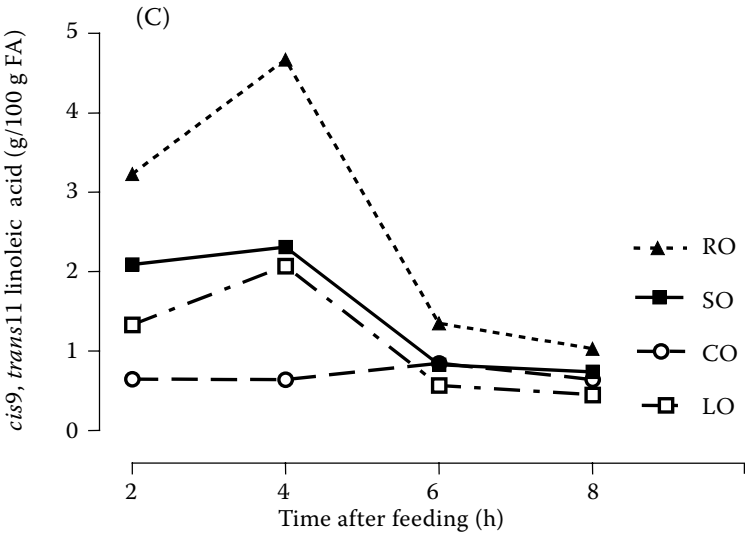
SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids



Oil: $P < 0.05$; Time: ns; Oils × Times: NS (S.E.M. = 3.5)



Oil: $P < 0.01$; Time $P < 0.05$; Oils × Times: NS (S.E.M. = 2.8)



Oil: $P < 0.001$; Time $P < 0.001$; Oils × Times: $P < 0.05$ (S.E.M. = 0.6)

Figure 1. (A–C) Profiles (2, 4, 6 and 8 h after feeding) of stearic acid (A), trans-vaccenic acid (B), and *cis9, trans*11 conjugated linoleic acid (C) in the rumen fluid of sheep fed diets supplemented with sunflower oil (SO), rapeseed oil (RO) or linseed oil (LO), control is CO

Table 2. The effect of oil supplements on different fatty acids (g/100 g FA) in the rumen fluid of sheep

Fatty acid, time after feeding (h)		Oil				Pooled S.E.M.	Significance		
		SO	RO	LO	CO		oil	time	oils × times
C16:0 palmitic	2	15.9	16.9	17.4	25.5	1.1	***	**	NS
	4	12.0	12.6	13.9	24.8				
	6	12.5	12.4	12.9	24.0				
	8	14.3	13.4	15.2	26.0				
cis C18:1*n*-9 oleic	2	5.52	6.01	4.61	4.03	0.5	**	**	NS
	4	4.88	5.46	3.54	3.65				
	6	3.60	3.85	2.74	3.60				
	8	3.71	4.08	3.08	3.30				
trans C18:2*n*-6 linoleic	2	0.20	0.17	1.13	0.88	0.3	***	NS	NS
	4	0.14	0.26	1.43	0.35				
	6	0.50	0.50	1.47	0.42				
	8	0.11	0.30	1.27	0.56				
cis C18:2*n*-6 linoleic	2	6.36	7.99	10.3	5.77	1.2	***	NS	NS
	4	3.03	3.91	11.3	5.74				
	6	2.62	2.67	12.1	6.70				
	8	2.76	2.67	10.2	6.89				
C18:3*n*-3 α-linolenic	2	0.86	1.09	5.05	2.29	0.2	***	NS	*
	4	0.63	0.69	3.23	2.45				
	6	0.74	0.68	2.59	2.79				
	8	0.93	0.73	2.34	2.98				

SO – sunflower oil; RO – rapeseed oil; LO – linseed oil; CO – control; FA – fatty acids; NS – not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

at program rate 34°C/min) with hydrogen as a carrier gas (flow 1.8 ml/min, velocity 44 cm/s, pressure 23.2 psi). The fatty acid methyl ester peaks were identified with a commercial mixture (Supelco 37 component FAME MIX, Supelco, Bellefonte, PA, USA; *trans*11-vaccenic (TVA) methyl ester, Supelco, Bellefonte, PA, USA; *cis*9, *trans*11 conjugated linoleic acid (c9,t11-CLA), Matreya, PA, USA) and quantified by an internal standard (tridecanoic acid C13:0, Supelco, Bellefonte, PA, USA). The values of fatty acids are expressed as g/100 g of total fatty acids (g/100 g FA). Statistical analysis of fatty acids: results were analyzed by analysis of variance (mixed model ANOVA; Graphpad InStat, GraphPad Software, Inc. San Diego, CA, USA). Effects included in the model were oil, time after feeding and interactions between parameters. Total and individual protozoan concentrations were analyzed by one-way analysis of variance using the Student-Newman-Keuls test (Graphpad InStat, GraphPad Software, Inc. San Diego, CA, USA).

RESULTS

The concentration of stearic acid was increased 2 h after feeding (LO) and 4 h after feeding (SO, RO) over time (Figure 1A). The RO and SO oil supplementation of the diet led to a higher ($P < 0.01$) TVA concentration relative to LO and control (Figure 1B). The concentration of this isomer with both SO or RO supplements was the highest 4 h after feeding and then decreased ($P < 0.05$). The c9,t11-CLA concentration increased with time (4 h after feeding; $P < 0.01$) in the case of each oil supplement with the highest concentration with RO (Figure 1C). The concentration of c9,t11-CLA was the highest 4 h after feeding with each oil supplement and then decreased ($P < 0.001$). No interactions of the oil supplements and time after feeding were detected in the concentration of palmitic acid, oleic acid, and trans or cis linoleic acid in the rumen fluid (Table 2). However, the interaction of the oil supplements and time after feeding was detected in the concentration of α-linolenic acid ($P < 0.05$). The content

Table 3. The effect of oil supplements on saturated and unsaturated fatty acids (g/100 g FA) in the rumen fluid of sheep

Fatty acids, time after feeding (h)		Oil				Pooled S.E.M.	Significance		
		SO	RO	LO	CO		oil	time	oils × times
SCFA	2	0.75	0.82	0.88	1.10	0.2	***	***	NS
	4	0.30	0.45	0.48	1.00				
	6	0.38	0.33	0.45	0.75				
	8	0.38	0.43	0.53	0.85				
MCFA	2	20.7	22.5	23.3	36.0	1.3	***	***	NS
	4	14.9	15.5	17.3	32.3				
	6	15.1	15.3	16.2	30.4				
	8	17.3	16.3	20.3	31.9				
LCFA	2	78.8	76.6	76.2	62.9	1.7	***	**	NS
	4	83.8	82.5	80.5	66.8				
	6	82.3	84.6	79.9	68.9				
	8	81.3	81.3	78.2	66.2				
UFA	2	63.1	43.8	47.6	21.8	2.0	***	***	***
	4	55.2	58.0	49.1	22.6				
	6	53.3	46.4	36.6	24.3				
	8	44.8	43.6	43.0	26.6				
SFA	2	39.4	54.9	54.1	76.9	2.0	***	***	***
	4	42.6	43.6	51.0	77.7				
	6	45.2	53.8	59.8	75.5				
	8	55.0	55.8	57.3	72.7				

SO – sunflower oil; RO – rapeseed oil; LO – linseed oil; CO – control; NS – not significant; $^{**}P < 0.01$; $^{***}P < 0.001$ SCFA – short-chain fatty acids (C4:0-C13:0); MCFA – medium-chain fatty acids (C14:0-C17:1); LCFA – long-chain fatty acids (C >18); UFA – unsaturated fatty acids; SFA – saturated fatty acids

of palmitic acid differed with the oil supplements ($P < 0.001$) and time after feeding ($P < 0.01$). Within all the oil treatments, the content of palmitic acid decreased 4 and 6 h after feeding and increased 8 h after feeding. The oleic acid concentration decreased all the time ($P < 0.01$) until 6 h after feeding with all the oil supplements. The concentration of oleic acid in the rumen fluid was higher ($P < 0.01$) with RO and SO compared with LO and CO ($P < 0.01$) 2 and 4 h after feeding. The content of trans and cis linoleic acid in the rumen fluid was higher ($P < 0.001$) with LO compared to SO, RO and CO. Within LO treatment, the content of α-linolenic acid in the rumen fluid was the highest 2 h after feeding compared to SO, RO and CO. Overall effects of the oil supplements on short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA), long-chain fatty acids (LCFA), unsaturated fatty acids (UFA), and saturated fatty acids (SFA) in the rumen fluid are shown in Table 3. The effect of oil and time after feeding on SCFA, MCFA, and LCFA was evident. However, the two-way interaction was not significant. Strong interaction of oil supplements and times after feeding was detected in the concentration of UFA and SFA ($P < 0.001$, Table 3). An adverse effect of LO on the total protozoan concentration ($P < 0.001$) and *Entodinium* spp. population ($P < 0.01$) was observed (Table 4). During the supplementation with LO the concentration of *Dasytricha ruminantium* ($P < 0.05$) and the population of *Isotricha* spp. ($P < 0.01$) were decreased. The concentration of *Isotricha* spp. with SO was lower ($P < 0.05$) compared to the control. The population of large Entodiniomorphids was decreased by LO (*Polyplastron multivesiculatum* $P < 0.05$, *Ophryoscolex c. tricoronatus* $P < 0.01$, *Eremoplastron dilobum* $P < 0.01$), by RO (*Polyplastron multivesiculatum* $P < 0.05$, *Eremoplastron dilobum*, $P < 0.01$), and by SO (*Eremoplastron dilobum* $P < 0.01$).

Table 4. Total and individual protozoan concentration (number/ml) in the rumen fluid of sheep fed diet with various oil supplements

	SO	RO	LO	CO	Pooled S.E.M.
Total protozoan concentration	75 430	72 000	50 180***	89 050	5 973
Entodinium spp.	67 350	64 300	44 960**	71 030	4 657
Dasytricha ruminantium	4 625	4 308	3 200*	7 150	830
Isotricha spp.	506*	589	451**	790	74
Diploplastron affinae	1 988	1 825	1 783	3 325	486
Polyplastron multivesiculatum	185	96*	83*	180	31
Ophryoscolex c. tricoronatus	111	105	53**	186	27
Eremoplastron dilobum	54*	33**	38**	83	9

SO – sunflower oil; RO – rapeseed oil; LO – linseed oil; CO – control; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ differences from control

DISCUSSION

Concentrations of TVA and c9,t11-CLA in the rumen fluid were increased with all three oil supplements and varied depending on the time after feeding and oil supplements in the diet as shown in Figure 1 B–C. During the biohydrogenation of polyunsaturated fatty acids, the concentration of linoleic and α-linolenic acid decreased as they are hydrogenated to stearic acid with the formation of intermediates such as CLA and TVA (Harfoot and Hazelwood, 1988). The concentration of stearic acid with SO and RO (Figure 1A) indicated a shift of the biohydrogenation of unsaturated fatty acids to the accumulation of TVA in the rumen. In contrast to SO and RO, the higher concentration of stearic acid with LO indicated a shift of the biohydrogenation of unsaturated fatty acids to a lower mean concentration of TVA. The accumulation of TVA is probably due to an excess of free fatty acids that inhibited the final hydrogenation of TVA to stearic acid (Gulati et al., 2000). According to Lock and Garnsworthy (2003), possible reasons for increases in the concentration of TVA include an increased intake of substrates (linoleic acid and α-linolenic acid) and/or a decrease in the final hydrogenation step from TVA to stearic acid in the rumen. A higher concentration of c9,t11-CLA in the rumen fluid in our experiment was observed with RO, ranging from 2.23 g/100 g FA (2 h after feeding) to maximum 4.67 g/100 g FA (4 h after feeding) whilst with SO and LO the maximum c9,t11-CLA concentration was 2.31 g/100 g FA and 2.07 g/100 g FA, respectively (4 h after feeding). The enhancement of *cis*9, *trans*11-C18:2 with SO in the rumen fluid of cows fed a high concentrate diet was reported by Loor et al. (2004a). The concentration of TVA and c9,t11-CLA with LO in our experiment was lower compared to RO and SO. The lower concentration of these isomers with LO was accompanied by the higher concentration of α-linolenic acid in the rumen fluid compared to SO, RO and CO. The input of α-linolenic acid when hydrogenation is incomplete may result in an enhanced ruminal outflow of *trans*11,*cis*15–linoleic acid, TVA and *trans*13–14 –15 isomers of oleic acid (Loor et al., 2004b). According to Loor et al. (2002) LO may increase an endogenous synthesis of c9,t11-CLA in tissues by enhancing the post-absorptive availability of TVA. It is evident that the differences in c9,t11-CLA concentration between oil supplements are influenced by the level of linoleic acid in the original oils used to produce CLA (Yu et al., 2003; Szöllöskei et al., 2005). Greater α-linolenic acid hydrogenation with LO and greater linoleic acid hydrogenation with SO were documented by Loor et al. (2005). A high concentration of linoleic acid in the diet would reduce biohydrogenation and increase the postruminal flow of this unsaturated fatty acid (Beam et al., 2000). Since most of the CLA excreted in the milk originated from TVA modification in the mammary gland tissue, it is very important what type of oils is used. The diet supplemented with 5% of LO decreased the protozoan population in comparison with the control in the rumen fluid of sheep in our experiment. Contrary to findings of Ivan et al. (2001) no significant effect of SO supplementation on the protozoan concentration was observed. It is well known that oils high in C18 fatty acids are toxic to protozoa (Newbold and Chamberlain, 1988; Ivan et al., 2003, 2004; Hristov et al., 2004). On the other hand, the

rumen ciliates could play an important role in the rumen content of TVA and CLA (Devillard et al., 2006; Yánez-Ruiz et al., 2006; Or-Rashid et al., 2007). Recently, Kišidayová et al. (2006) suggested that the rumen ciliates had no uniform response to oil supplements in *in vitro* studies.

In conclusion it can be said that the highest concentrations of TVA and c9,t11-CLA in the rumen fluid were with SO and RO without major changes in the rumen ciliate population. Fat supplements for ruminants may also interact with the protection of animal health and the environment. From this aspect, the use of various oils at optimum levels in diets of sheep can be useful in the agricultural practice.

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