Modification of milk fatty acid composition by feeding forages and agro-industrial byproducts from dry areas to Awassi sheep

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ABSTRACT

The study tested the hypothesis that certain under-used forages and agro-industrial byproducts available in dry areas may positively influence fatty acid (FA) composition and antioxidative properties of milk by their contents of residual oil or phenolic compounds or both. Sixty multiparous fat-tailed Awassi ewes were allocated to 6 groups in a completely randomized block design. During 50 d, the ewes were group-fed 2.5 kg of dry matter/d per ewe 1 of 6 isonitrogenous and isoenergetic diets (forage:concentrate, 0.3:0.7). The test feeds, comprising 30% of the diets, replaced either barley straw [lentil straw, olive leaves, and *Atriplex* (saltbush) leaves, rich in phenolic compounds or electrolytes] or conventional concentrate ingredients (olive cake and tomato pomace; ~10% lipids) from the control diet. The diets containing olive cake and tomato pomace were rich in oleic acid (18:1 *cis*-9; 27% of total dietary FA) and linoleic acid (18:2 *cis*-9, *cis*-12; 37%), respectively. Profiles of FA were determined in individual milk samples drawn on d 0 and in wk 1, 3, 5 and 7. Data was analyzed by repeated measurement analysis. No consistent treatment effects on yield and gross nutrient composition of the milk were observed, although some differences occurred. Milk resulting from the *Atriplex* leaf diet expressed the highest antiradical activity, which was low with control and olive leaves. Feeding the tomato pomace and olive cake diets decreased the proportions of short- and medium-chain FA, whereas oleic acid clearly increased in proportion to total FA. Olive leaves most effectively increased rumenic acid (18:2 *cis*-9, *trans*-11) and α -linolenic acid (18:3 *cis*-9, *cis*-12, *cis*-15) in milk fat. This also resulted in the highest α -linolenic acid transfer rate from feed to milk and suggests that olive leaves affect ruminal biohydrogenation at several steps. Several alternative feeds exist with an added value, as they enhance FA with potential health

benefits and the stability of the milk with higher antioxidative activity, even though responses to test feeds differed largely. It remains to be investigated whether combinations of these feeds would be complementary in these beneficial effects.

Key words: forage, byproduct, fatty acid, Awassi

INTRODUCTION

Large sheep and goat populations and adverse climatic conditions cause feed shortages, thus limiting small ruminant productivity and farm income in the dry areas of the Mediterranean basin. The inclusion of underused shrubs and local agro-industrial byproducts in the diets, as alternatives to straw and cereal-based concentrates, is discussed as a potential solution (Vasta et al., 2008). However, such feeds often contain anti-nutritive compounds, which may decrease both their palatability and digestibility (Makkar, 2003a). A low nutritional value makes their use in high-yielding dairy cows inefficient (Chiofalo et al., 2004), whereas local breeds of dairy ewes have developed specific mechanisms for adaptation (Lanzani et al., 1993), allowing them to better cope with suboptimal diets. Research on alternative feeds is mostly restricted to the evaluation of their nutritional value and effects on animal performance. Only recently, some work has been conducted on changes in milk fatty acid (FA) composition when feeding olive cake (Chiofalo et al., 2004), olive leaves (Tsiplakou and Zervas, 2008) and tomato pomace (Romano et al., 2010), but this did not include a comparative evaluation of these feed alternatives.

Dietary FA composition determines the FA profile in milk; this even in ruminants, where the polyunsaturated FA (PUFA) are subjected to intensive microbial biohydrogenation, (Bauman and Griinari, 2003). The FA of interest for human health in the milk from ruminants include n-3 PUFA (Kromhout et al., 2010), among them α -linolenic acid [18:3 *cis*(c)-9, c12, c15]. The n-3 PUFA with more than 18 carbon atoms can be derived in man in small amounts from chain elongation and further desaturation of 18:3 c9, c12, c15 (Burdge, 2006). Ruminal biohydrogenation of dietary PUFA also pro-

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duces nutritionally relevant FA (Chilliard et al., 2007). Here, the focus is on rumenic acid [18:2 *c9,trans(t)*-11], a conjugated linoleic acid (CLA) isomer, and vaccenic acid (18:1 *t*11; Goudjil et al., 2004, 2007). The content of these FA in milk fat depends on the intake of their precursors and the degree to which the precursors are transformed during the ruminal biohydrogenation (Lourenço et al., 2008).

The composition of the diet can also influence the concentration of fat-soluble micronutrients in the milk, in particular β -carotene and α -tocopherol (Martin et al., 2004), and plant secondary metabolites like phenolic compounds (O'Connell and Fox, 2001). These compounds are involved in the prevention of the photo-oxidation of the milk fat globule membrane, the major site of auto-oxidation (Lindmark-Månsson and Åkeson, 2000; O'Connell and Fox, 2001). The occurrence of these active principles in milk can also increase its nutritional and organoleptic quality (O'Connell and Fox, 2001; Nozière et al., 2006).

The present study tested the hypothesis that, in response to their particular composition, certain agro-industrial byproducts and forages may influence milk fat properties in a favorable way. Test feeds included tomato pomace and olive cake, both rich in lipids, olive leaves and lentil straw, both rich in polyphenols, and the salty leaves of the *Atriplex* bush. The feeds were obtained from a typical Mediterranean production area in Syria and their palatability, digestibility, and energy content has been determined in earlier experiments (Abbeddou et al., 2011a,b). In the current study, ewes of the Awassi breed, a fat-tailed dairy genotype adapted to the specific feeding and environmental conditions, were fed a diet that contained 30% of the test feeds. A special emphasis was put on the milk FA profile, FA transfer from feed to milk, and the antioxidant status of the milk.

MATERIALS AND METHODS

Experimental Design

The experiment was conducted at the International Center of Agricultural Research in the Dry Areas (ICARDA, Aleppo, Syria). Sixty Awassi ewes in their second to fourth lactation, weighing, on average, 51.0 ± 6.8 kg were kept indoors and allocated to 6 dietary treatment groups in a completely randomized block design. At the start of the experiment, the sheep were 67.2 ± 10.0 DIM, after the lambs had been allowed to suckle for 2 mo, consistent with common practice in Syria. Additionally to these 2 criteria, the groups were also balanced for individual milk yield, as well as milk fat and milk protein contents.

Diets and Feeding

Three forages and 2 agro-industrial byproducts were tested in comparison with a barley-straw concentrate-based control diet, which consisted of ingredients traditionally used in Syria. Lentil (*Lens culinaris*) straw was purchased from a market in Aleppo. Fresh branches from saltbush (*Atriplex halimus*) were collected from the steppe in the Adameh area. Olive (*Olea europea* L.) branches from routine pruning of the trees were purchased on the Afrin market in Aleppo. The tomato pomace originated from the tomato purée factory Conserva (Edleb, Syria). The residue from the first press of green olives, which included peel and kernels, was obtained from a traditional cold pressing olive oil factory in Afrin. The fresh leaves and tomato pomace were immediately sun-dried for 3 to 4 d, whereas olive cake was oven-dried at 40°C for 4 d at ICARDA's facilities. Afterward, leaves with small twigs of olive and *Atriplex* were separated by hand from larger branches. All feeds had been obtained as batches for the entire experiment and batches were the same as those that had been evaluated in earlier experiments (see more details in Abbeddou et al., 2011a,b).

All test feeds constituted 30% of DM of the complete diets (Table 1). Diets were balanced for their contents of energy as measured previously (Abbeddou et al., 2011a,b) and CP. For the latter purpose, some urea was added to the concentrates. Diets were composed of forage and concentrate in a ratio of 30:70, except for the olive cake diet with a ratio of 20:80. Barley straw, as the control forage, was completely substituted by lentil straw, *Atriplex*, or olive leaves in the respective diets. Proportionately, 3/7 of the control concentrate, mainly consisting of sugar beet pulp, wheat bran, and cottonseed meal, was replaced by olive cake or tomato pomace. The limited energy content of these feeds required that also wheat grain was used to replace wheat bran and all or most of the sugar beet pulp. Molasses was added to all diets to improve palatability (mixed with olive leaves, *Atriplex* leaves, and the concentrate for the other diets). All groups received 20 g/d per head of a vitamin-mineral mix which was mixed with the concentrate. Diets containing tomato pomace and olive cake were rich in lipids (ether extract and total FA) compared with the other diets (Table 2). The lipids of the control diet were characterized by high proportions of saturated FA, followed by linoleic acid (18:2 *c9,c12*; n-6). Linoleic acid was the main FA in the tomato pomace and lentil straw diets, and the PUFA proportion was high. In olive cake, oleic acid (18:1 *c9*; n-9) was particularly prominent, resulting in an equal proportion of 18:1 *c9* and 18:2 *c9,c12* in the olive cake diet, where these 2 FA represented more than half of the total FA.

Table 1. Ingredients of the experimental diets (% of DM)¹

Item	Experimental diet					
	Control	Lentil straw	<i>Atriplex</i> leaves	Olive leaves	Olive cake	Tomato pomace
Test feed	—	30	30	30	30	30
Barley straw	30	—	—	—	20	30
Wheat grain	—	—	—	—	30	20
Sugar beet pulp	30	20	30	30	—	5
Wheat bran	10	20	20	20	—	—
Cottonseed meal	20	20	10	10	10	5
Molasses	10	10	10	10	10	10

¹Diets were individually supplemented with 20 g of mineral-vitamin mix/d per head, containing per kilogram: Ca, 138 g; P, 108 g; Mg, 151 mg; Na, 123.5 g; Cu, 119 mg; Mn, 774 mg; I, 76 mg; vitamin A, 12,500,000 IU; vitamin D₃, 300,000 IU; vitamin E, 0.5 g; and urea at levels of 8, 0, 5, 12, 19, and 16 g/kg of DM for control, lentil straw, *Atriplex* leaves, olive leaves, olive cake, and tomato pomace diets, respectively.

At much lower total fat content, the olive leaf diet was especially rich in 18:3 *c*9,*c*12,*c*15. This diet was richer in total phenols and total tannins than the other diets, followed by the lentil straw diet. The tomato pomace and olive cake diets were rich in lignified fiber, whereas fiber content was low in the *Atriplex* leaf diet.

Before the experiment started, the ewes had been fed 1.3 kg of DM/head of a mixed concentrate (barley grain, cottonseed meal and sugar beet pulp, 50:25:25) and grazed natural pasture at an approximate biomass intake of 0.7 kg of DM/d per head. On experimental d 0 and 1, 50 and 75% of this concentrate was replaced by the experimental concentrates and grazing was omitted. At the same time, barley straw and the other experimental forages (1:1) were offered in replacement of the pasture grass. From d 2 onwards, the ewes were exclusively fed the experimental diets, which were offered at increasing levels until reaching 2.5 kg of DM/d per head after 10 d. These 10 d were part of the entire 50 d of the experiment. Eventually, per group, 0.75 kg of forage and 1.75 kg of concentrate per head per day were offered, except for the olive cake group, in which 0.5 kg of forage and 2.0 kg of concentrate were offered per head per day to achieve that this diet was also isoenergetic to the other diets. Half of the daily portions were provided at 0800 and 1700 h always directly after milking. Water was offered ad libitum.

Data Recording and Sample Collection

Samples of the dietary ingredients were drawn for analysis 3 times during the experiment. Body weight was measured at the beginning of the experiment and then every second week using a balance with 1-kg increments. This was done after milking and before feeding. Milking was performed daily starting at 0600 and 1600 h by a machine milking system; however, for the weekly recordings of daily milk yield and for milk sampling, milking was done manually, starting at the same time

as machine milking. The weekly collected morning milk samples were immediately subjected to constituent composition analysis. Additional samples obtained on d 0 and wk 1, 3, 5, and 7 of the experiment were immediately stored at –20°C for analysis of anti-radical activity and FA profile.

All procedures involving the animals followed the international guiding principles listed by the Council for International Organizations of Medical Sciences (2010).

Gross Nutrient Analysis in Feeds and Milk

Feeds were analyzed for their proximate contents by standard methods (AOAC, 1997). These included DM and total ash (AOAC index no. 942.05), CP (AOAC 977.02; using Kjeldahl unit Vapodest 30; C. Gerhardt GmbH & Co. KG, Königswinter, Germany), and ether extract (**EE**; AOAC 963.15; Soxtherm Variostat; C. Gerhardt Ltd., Northamptonshire, UK). Contents of NDF, ADF, and acid detergent lignin were determined in feeds corrected for ash content. In the case of NDF, α -amylase and sodium sulfite were used (Van Soest et al., 1991). Nonfiber carbohydrates were calculated as OM – (NDF + CP + EE). Additionally, total phenol and total tannin (Folin-Ciocalteu method; Makkar, 2003b), as well as condensed tannin, contents (Porter et al., 1986) were determined. Total phenol and total tannin contents were expressed as tannic acid equivalents and condensed tannins as leucocyanidin equivalents.

Contents of fat, protein, lactose, TS, and SNF in milk were measured by the Milkoscan 133 B (Foss Electric, Hillerød, Denmark). The ECM (kg/d) was calculated based on the ALP (2008) formula as milk (kg/d) $\times$ $[0.38 \times \text{fat} (\%) + 0.24 \times \text{protein} (\%) + 0.17 \times \text{lactose} (\%)]/3.14$.

Analysis of the Antiradical Activity of the Milk

The antiradical effect of individual milk samples on the free radical 2,2-diphenyl-1-picrylhydrazyl (**DPPH**)

Table 2. Nutrient composition (% of DM) of the different experimental feeds and diets as offered (n = 3)

Item ¹	Lentil straw			<i>Atriplex</i> leaves		Olive leaves		Olive cake		Tomato pomace	
	Control	Feed	Diet	Feed	Diet	Feed	Diet	Feed	Diet	Feed	Diet
OM	89.7	89.0	89.6	69.0	83.4	91.1	89.3	86.4	87.5	95.2	89.8
CP	17.4	9.3	17.6	16.1	17.6	9.4	17.5	7.9	18.4	19.1	18.1
NDF	41.5	42.7	32.1	25.8	29.3	27.8	29.7	58.4	42.8	55.2	46.3
ADF	24.3	30.5	21.4	16.5	18.1	22.8	19.8	45.9	23.4	46.2	27.1
ADL	3.88	7.27	4.79	7.93	4.68	10.94	5.53	23.74	8.64	25.9	9.47
Non-NDF carbohydrates	32.0	32.7	37.5	25.1	36.4	44.4	41.9	10.9	28.6	10.9	26.6
Ether extract	1.04	1.49	1.52	1.40	1.33	3.15	1.84	9.22	3.07	9.99	3.34
Total FA	1.97	0.78	2.49	0.48	1.80	0.78	1.87	5.91	2.98	13.36	4.74
FA (% of total FAME)											
16:0	25.5	19.8	19.8	28.6	22.9	24.7	21.6	17.5	19.2	14.5	20.5
16:1	0.67	0.26	0.50	0.38	0.60	0.46	0.62	0.59	0.39	0.33	0.38
18:0	2.63	4.83	2.57	3.27	2.02	2.62	1.81	4.98	2.97	4.59	3.22
18:1 <i>c</i> 9	14.2	11.5	15.2	10.0	14.6	12.7	15.3	64.2	27.0	20.7	13.7
18:2 <i>c</i> 9, <i>c</i> 12	29.8	27.9	35.1	15.4	29.3	11.1	27.8	6.6	27.0	55.1	36.5
18:3 <i>c</i> 9, <i>c</i> 12, <i>c</i> 15	2.4	11.6	4.9	14.4	6.0	25.7	9.3	0.3	2.0	2.6	2.8
SFA	39.2	41.5	30.0	53.0	34.6	40.2	30.6	25.2	28.6	20.4	32.6
MUFA	16.9	18.3	18.9	15.6	18.3	22.3	20.1	67.7	29.8	21.8	15.9
PUFA	32.5	40.2	40.4	31.4	36.0	37.5	37.5	7.1	29.2	57.7	39.4
Total phenols ²	0.67	2.80	1.32	0.63	0.57	6.35	2.25	0.41	0.53	0.64	0.60
Total tannins ²	0.37	1.94	0.84	0.32	0.28	3.16	1.12	0.17	0.25	0.40	0.31
Condensed tannins ³	0.10	0.44	0.22	0.00	0.05	0.05	0.07	0.00	0.05	0.00	0.04

¹ADL = acid detergent lignin; FA = fatty acids; FAME = FA methyl esters; *c* = *cis*; SFA = saturated FA; MUFA = monounsaturated FA; PUFA = polyunsaturated FA.

²Tannic acid equivalents.

³Leukocyanidin equivalents.

was measured using a slightly modified version of the protocol of Liu et al. (2005). Briefly, 75 μL of the thawed milk was added to 1,425 μL of DPPH solution (6×10^{-5} M dissolved in methanol), vortexed, and stored in the dark for 30 min. Then the absorbance at 515 nm was measured using a UV-Vis spectrophotometer (model U-2000; Hitachi, Tokyo, Japan). Methanol was used for determining the baseline value. The absorbance of the DPPH radical without sample was used as a control. The antiradical activity is defined as the ability of the milk to scavenge DPPH free radicals, given in percent and calculated as $(1 - \text{absorbance of the sample at 515 nm} / \text{absorbance of the control at 515 nm}) \times 100$.

Fatty Acid Analysis

Lipid extraction from the milled feed samples was done using a hexane-isopropanol solution (3:2). Amounts of 2 to 2.5 g were weighed into extraction thimbles and lipids were extracted overnight in an accelerated solvent extractor (ASE200; Dionex Corp., Sunnyvale, CA). As an internal standard, 11:0 (triglyceride, Fluka; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was added. The solvent was then evaporated under an N_2 stream with a TurboVap LV Evaporator (Zymark Center, Hopkinton, MA). Residues were dissolved in dichloromethane and evaporation under N_2 was repeated. Conversion of FA to FA methyl esters (**FAME**) was carried out according to method 2.301 of IUPAC (1987) with slight modification. The extract was boiled with 2 mL of NaOH (0.5 M) for 3 min, before 3 mL of methanolic boron trifluoride (1.3 M) was added. The mixture was heated again for 4 min and the reaction was terminated by adding 7 mL of NaCl (0.34 M) and 1 mL of hexane. Tubes were shaken for 30 s and centrifuged at $894 \times g$ for 6 min. Subsequently, 0.5 mL of the upper phase was purified on a column filled with silica gel (Merck KGaA, Darmstadt, Germany). The FAME were eluted with 6.5 mL of dichloromethane. These residues were injected into a gas chromatograph (HP 6890; Hewlett-Packard, Wilmington, PA) equipped with a Supelcowax-10 column (30 m $\times$ 0.32 mm $\times$ 0.25 μm ; Supelco Inc., Bellefonte, PA). Amounts of 1 μL were applied by split injection (1:30) at an injection temperature of 260°C. The carrier gas was H_2 , flow rate was 2.2 mL/min, and pressure was 55.8 kPa. The following temperature program was used: start at 160°C for 0.5 min; increase by 20°C/min up to 190°C; increase by 7°C/min up to 230°C; isotherm at 230°C for 5.3 min; increase by 20°C/min up to 250°C; isotherm for 6 min. Fatty acids were identified and quantified with the HP ChemStation software (Hewlett-Packard, Wilmington, NC), by comparison with other chromatograms, run under the same conditions (Collomb and Bühler, 2000)

and with reference to an external FAME standard (Supelco 37 component FAME mix; Supelco Inc.). The FAME proportions were adjusted by the response factors of the FAME standard and the internal standard (11:0), and their quantity in feed was calculated based on the internal standard and the feed sample weight.

Milk samples were thawed in a water bath at 37 °C and gently mixed to homogenously distribute the milk fat. The FA were extracted and analyzed according to the slightly modified method of Molkentin and Precht (2000). Briefly, 1 mL of milk was mixed with 2 mL of methanol and 1 mL of chloroform and then vortexed for 1 min. Another milliliter of chloroform and 1 mL of distilled water were added to the tube and vortexed again for better phase separation. The lower phase was carefully extracted and evaporated under N_2 . Hexane (1 mL) was added to the vial, which was then mixed with 20 μL of sodium methylate solution (2 M in methanol), shaken vigorously for 3 min, and centrifuged at $493 \times g$ (Kubota 3110; Kubota Corp., Tokyo, Japan) for 1 min. Thereafter, 100 mg of sodium hydrogen sulfate-monohydrate was added and the vial was recapped, vortexed again for 2 min, and centrifuged at $493 \times g$ for 1 min. One microliter of the clear supernatant was manually injected into the gas chromatograph (GC 2010; Shimadzu Corp., Kyoto, Japan) equipped with a highly polar (90% biscyanopropyl/10% phenyl cyanopropyl polysiloxane) capillary column (105 m $\times$ 0.25 mm i.d., 0.20- μm film, Rtx-2330; Supelco Inc.), a split-injection port and flame-ionization detector. Injector and detector temperatures were maintained at 250°C. As carrier gas, helium was used with a flow rate of 1.30 mL/min (linear velocity control). The initial oven temperature was set to 50°C for 3 min. and then the following temperature program was used: increase by 5°C/min up to 140°C; isotherm for 2 min; increase by 2°C/min up to 170°C; isotherm for 5 min; increase by 10°C/min up to 220°C; isotherm for 5 min; increase by 5°C/min up to 225°C; isotherm at 225°C for 20 min. Fatty acids were identified and quantified with the GC Solution program version 2.3 (Shimadzu Corp.) and by comparison with other chromatograms obtained under similar conditions (Collomb and Bühler, 2000). The response factor of the FAME standard was used to correct FAME proportions. Their quantity in the milk fat was calculated based on the fat content of the milk. Total 18:1 *cis* was calculated as the sum of 18:1 *c9*, *c11*, *c12*, *c13*, and *c14*; total 18:1 *trans* was the sum of 18:1 *t4* to *t12*, and total CLA was the sum of 18:2 *c9*, *t11*, *c9c11*, and *t9t11*.

Statistical Analysis

Data were analyzed performing repeated measurement analysis using the MIXED procedure of SAS

(version 9.2; SAS Institute, 2009) and assuming a compound symmetric structure. Effects considered were diet, week, and the interaction between the 2 factors, as well as the initial records measured at d 0 (covariate). The least squares means were statistically compared with the Tukey method both for diet and week. The same model was used for Figures 1 and 2, except that the initial records at d 0 were not used as a covariate.

RESULTS

Performance

Feed intake was similar among the different test diets and the control diet, and almost no refusals were recorded. On average, ewes of all groups gained weight during the experiment. The ADG (g) were high with control and lentil straw (257), followed by *Atriplex* (235), with the diets containing tomato pomace, olive leaves and olive cake being lowest (193, 180, and 175, respectively; treatment effect, $P = 0.037$). Milk yield, expressed as ECM, was highest with the control diet and lowest with olive leaves ($P = 0.005$; Table 3), whereas the other diets did not cause milk yield to differ significantly from either the control or the olive leaf diet.

Gross Nutrient Composition of Milk

The milk fat content was unaffected by diet except for the lentil straw diet where it was 9.0% lower compared with the control ($P = 0.029$) and the olive cake diet ($P = 0.023$; Table 3). The milk total protein content was high with lentil straw and low with tomato pomace ($P = 0.003$). The lactose content was highest with olive cake and lowest with the olive leaf and the control diet ($P < 0.001$). The diet type did not influence TS content, which first increased to reach the maximum from wk 3 to 4 and then decreased ($P < 0.001$) with progressing lactation (data not shown). The SNF contents were high with *Atriplex* leaves and lentil straw and low with tomato pomace ($P < 0.01$). No interaction between diet and week was observed for any compositional variable.

Antiradical Activity

Antiradical activity was highest in the *Atriplex* leaf diet (1.2-fold of the control antiradical activity) and lowest (0.95-fold of control) with the milk produced from the olive leaf diet ($P < 0.001$; Table 3).

Fatty Acid Composition of the Milk Fat

The diets containing olive cake and tomato pomace led to clearly lower 16:0 and higher 18:0 proportions

in milk fat compared with all other diets ($P < 0.001$; Table 4). Compared with the control, the proportion of 18:0 was lower with *Atriplex* leaves ($P = 0.018$) and especially with olive leaves ($P < 0.001$). The same effect as for 18:0 was observed for 18:1 *c9* ($P = 0.013$) and total 18:1 *cis* ($P = 0.031$) with the olive leaf diet. Total 18:1 *trans* FA proportions, including 18:1 *t10* and 18:1 *t11*, were enhanced by lentil straw and olive leaves. The proportion of 18:2 *c9,c12* did not significantly differ among the test forages and control diet, but decreased ($P < 0.001$) with tomato pomace and olive cake. The total CLA proportions were highest ($P < 0.001$) in milk fat from the olive leaf and the tomato pomace diets, low with olive cake and control diets, and intermediate with lentil straw and *Atriplex* leaves. This was the same for the most prevalent CLA isomer, 18:2 *c9,t11*, but not for 18:2 *c9,c11* and 18:2 *t9,t11* (not displayed in Table 4). Compared with the control, the olive leaf diet promoted 18:3 *c9,c12,c15* to an almost 3-fold level, and proportions were also high with this diet for the long-chain n-3 PUFA; 20:5 *c5,c8,c11,c14,c17* (2.1-fold of the control); 22:5 *c7,c10,c13,c16,c19* (1.4-fold of control); and 22:6 *c4,c7,c10,c13,c16,c19* (1.3-fold of the control). Next to the olive leaf diet, diets with lentil straw and *Atriplex* leaves promoted a higher proportion of the n-3 FA compared with the control (mostly significant). The n-6:n-3 FA ratio was lower ($P < 0.001$) with olive leaves than with tomato pomace and the control, with the other dietary treatments being intermediate. The olive leaf diet also increased ($P = 0.019$) the proportion of the short-chain FA (SCFA, 4:0 to 11:0) compared with the control. The proportion of medium-chain FA (MCFA; 12:0 to 16:1) was similar for the control, olive leaf, and *Atriplex* leaf diets and lower ($P < 0.001$) with tomato pomace and olive cake. The latter diets, at the same time, increased ($P < 0.001$) monounsaturated FA proportions at a cost of the saturated FA. The proportion of the PUFA was low with olive cake ($P < 0.001$) and high with olive leaves and lentil straw ($P < 0.001$ compared with the control). The statistical analysis showed an effect of the experimental week ($P < 0.001$) on most of the milk FA.

Fatty Acid Excretion with Milk

The daily excretion of the main PUFA and those FA, which are, at least partly, the result of their biohydrogenation, were mostly adapted to the new levels after 1 or 3 wk of experimental feeding (Figures 1 and 2). Thereafter, changes with time were small except for the two 18:1 *trans* FA (*t10* and *t11*) displayed. As effects of the diets on milk fat yield were not very large, excretion of individual FA was similarly affected by the experimental diets as the proportions of these FA of

Figure 1. Evolution of the daily excretion of the 2 major polyunsaturated fatty acids [linoleic acid (18:2 c9,c12; n-6) and α -linolenic acid (18:3 c9,c12,c15)] and their most important biohydrogenation products with the milk as affected by diet. Δ , control; $\blacktriangle$, lentil straw; $\square$, *Atriplex* leaves; $\blacksquare$, olive leaves; $\circ$, olive cake; $\bullet$, tomato pomace. c = cis; t = trans.

total FAME. Diet differences were especially obvious for 18:3 c9,c12,c15 (high with the olive leaf and *Atriplex* leaf diets), 18:1 c11 (high with the olive leaf diet), and 18:1 c9 and 18:0 (both high with the olive cake and tomato pomace diets; Figures 1 and 2).

Fatty Acid Recovery Rates

When compared with their ingested amounts, the dietary FA, which are subject to modification by ruminal biohydrogenation (18:3 c9,c12,c15, 18:2 c9,c12 and 18:1

c9) were largely disappearing from feed to milk, whereas the terminal end product of biohydrogenation, 18:0, increased to levels that were 1.8- to 4.7-fold of those ingested (Table 5). Recoveries of 18:3 c9,c12,c15; 18:2 c9,c12; and the increase in 18:0 were small with tomato pomace and large with the control and *Atriplex* leaf diets ($P < 0.001$). Even though the olive cake diet was rich in 18:1 c9 (27.0% of total dietary FAME), which was well reflected in the milk fat (20.4%), the recovery rate of 18:1 c9 was lowest with this diet ($P < 0.001$).

Figure 2. Evolution of the daily excretion of stearic acid (18:0) and oleic acid (18:1 *c*9) with the milk as affected by diet. Δ , control; $\blacktriangle$, lentil straw; $\square$, *Atriplex* leaves; $\blacksquare$, olive leaves; $\circ$, olive cake; $\bullet$, tomato pomace. *c* = cis.

DISCUSSION

As intended by the isoenergetic and isonitrogenous formulation of the diets, differences in performance were not very large. The comparably low ECM yield and weight gain with progressing lactation when feeding olive leaves and olive cake indicated that ME content may have been slightly over-estimated in the preceding digestibility experiments (Abbeddou et al., 2011a,b). It seems that adverse factors like the high acid detergent lignin:NDF ratio (olive leaf, olive cake, and tomato pomace diets), the relatively high content of phenols (olive leaf diet), and the high EE content (olive cake and tomato pomace diet) were more detrimental in lactating sheep with a higher requirement than in juvenile sheep approaching adulthood. Feeding the test feeds to small ruminants in other studies resulted in a decrease (olive cake; Molina-Alcaide et al., 2010), an unchanged (ammonia-treated olive leaves; Fegeros et al., 1995; tomato pomace; Romano et al., 2010), or even an increased milk yield (olive cake; Chiofalo et al., 2004). This discrepancy in results is likely related to the attempt and accuracy achieved in balancing for energy and nutrients needed to cover requirements of the respective animal species and breeds used.

Effects of the different diets on gross composition of milk were also limited in extent. Only the 2 diets with relatively high EE content were able to maintain the same milk fat content as that of the control, whereas milk protein contents tended to be lower. It is known that lipid supplementation in dairy sheep may increase milk fat and, at the same time, decrease protein content (Gómez-Cortés et al., 2008) as a consequence of lower rumen microbial protein synthesis. The comparatively low milk fat content, found with all test forages and especially with lentil straw, despite the fact that the latter was even more fibrous than the control diet, seems

puzzling at first glance. However, the high prevalence of total 18:1 *trans* FA and especially 18:1 *t*10 in milk fat was obviously associated with a certain inhibition of milk fat synthesis (Bauman and Griinari, 2003; Gómez-Cortés et al., 2008).

Antiradical Activity

A high antioxidative potential of human food is desired to reinforce the natural defense system against forming free radicals and initiating detrimental oxidative events in the metabolism (Parr and Bolwell, 2000). The main antioxidative compounds in milk are enzymes, lactoferrin, vitamins C and E, carotenoids (Nozière et al., 2006), and, to a certain extent, phenolic compounds (O'Connell and Fox, 2001). To test their efficiency, many *in vitro* and *in vivo* tests have been developed of which the simple free radical DPPH scavenging activity test has been used widely (Liu et al., 2005). Milk from the *Atriplex* leaf diet showed the highest antiradical activity, followed by diets containing tomato pomace, olive cake, and lentil straw. These diets have different potential active secondary metabolites like lycopene in tomato pomace and phenols in olive cake and *Atriplex* leaves, and it can be assumed that a transfer of some of these metabolites from feed to milk occurred. According to O'Connell and Fox (2001), phenolic compounds can be found in considerable amounts in ruminant milk, of which the majority originates from feed. It has to be stated that by using methanol as the solvent for the DPPH, polar active compounds like phenols would be the only reactants. Other nonpolar constituents might have been underestimated in their importance. Unexpectedly, the test diet with the highest content of total phenols (olive leaves) led to the lowest antiradical activity of the milk. However, with this diet, milk fat also was highest in 18:3 *c*9, *c*12, *c*15 and total PUFA (i.e., FA

Table 3. Effect of diet and experimental week on milk yield and composition (n = 10)

Item	Experimental diet						P-value	
	Control	Lentil straw	Atriplex leaves	Olive leaves	Olive cake	Tomato pomace	SEM	Diet × week
ECM (kg/d)	1.73 ^a	1.61 ^{ab}	1.65 ^{ab}	1.42 ^b	1.54 ^{ab}	1.63 ^{ab}	0.025	0.009
Composition (%)								
Fat	7.03 ^a	6.43 ^b	6.50 ^{ab}	6.64 ^{ab}	7.04 ^a	6.86 ^{ab}	0.060	0.003
Protein	6.05 ^{ab}	6.20 ^a	6.13 ^a	6.18 ^a	5.92 ^{ab}	5.74 ^b	0.035	<0.001
Lactose	4.58 ^b	4.74 ^{ab}	4.84 ^a	4.60 ^b	4.93 ^a	4.84 ^a	0.022	<0.001
TS	18.4	18.1	18.2	18.1	18.6	18.1	0.08	0.359
SNF	11.3 ^{ab}	11.6 ^a	11.7 ^a	11.5 ^{ab}	11.5 ^{ab}	11.3 ^b	0.03	0.003
Antiradical activity ¹ (%)	25.8 ^{bc}	27.6 ^{abc}	30.0 ^a	24.5 ^c	28.8 ^{ab}	29.0 ^{ab}	0.32	<0.001

^{a-c}Mean values within the same row sharing no common superscript are significantly different ($P < 0.05$).

¹The ability of the tested milk to scavenge the 2,2-diphenyl-1-picrylhydrazyl free radical.

with a high inclination to be oxidized). Therefore, part of the antiradical active agents might have immediately been spent in this case.

Important Fatty Acids and Groups of Fatty Acids in Milk Fat

Short- and Medium-Chain Fatty Acids. The SCFA and MCFA in milk are mainly synthesized de novo (almost all 4:0 to 14:0 and more than 50% of 16:0). The de novo synthesis process can be inhibited partly by long-chain FA (from diet or body fat mobilization) and by *trans* FA (especially 18:1 *t*10 and 18:2 *t*10,*c*12) originating from hydrogenated dietary fat or CLA supplements. The latter are also products of ruminal biohydrogenation (Bauman and Griinari, 2003; Chilliard et al., 2007), but typically are less prevalent than 18:1 *t*11 and 18:2 *c*9,*t*11, especially in grass- or hay-based diets (Leiber et al., 2005). In the present study, SCFA and MCFA levels did not seem to coincide with the ratio of 18:1 *t*10 to 18:1 *t*11, though. As expected, the olive cake and tomato pomace diets, containing the most EE and long-chain FA, effectively decreased both SCFA and MCFA contents in milk. Lentil straw, rich in 18:2 *c*9,*c*12 was far less efficient, but 16:0 content in milk fat also decreased to some extent. Contrary to the present results, Tsiplakou and Zervas (2008) found lower contents of SCFA and MCFA in the milk fat of olive leaf-fed ewes. In general, it cannot be totally excluded that shifts in SCFA and MCFA were at least partly the result of shifts in other FA in milk fat.

Oleic Acid and Stearic Acid. Milk 18:1 *c*9 is mostly generated by mammary Δ^9 -desaturation of stearic acid (18:0) but can also be transferred from feed to milk to a certain extent (Chilliard et al., 2007). Following the Mediterranean diet health claim, 18:1 *c*9 has gained attention (Chilliard et al., 2007). Two of the diets resulted in an almost 2-fold 18:1 *c*9 proportion in milk fat compared with all other diets. In the case of the olive cake diet this can be explained by the high dietary 18:1 *c*9 level, probably combined with slow ruminal biohydrogenation (Chiofalo et al., 2004). In the case of the tomato pomace, which was not especially rich in 18:1 *c*9, it can be assumed that 18:0 accumulated in the mammary gland, leading to correspondingly more 18:1 *c*9, resulting from an unchanged desaturation rate. Indeed, the tomato pomace diet contained much more 18:0 than the other diets except that with olive cake, and another important source of the 18:0 arriving at the mammary gland likely was biohydrogenation of the ample available dietary 18:2 *c*9,*c*12, the transfer of which from feed to milk was lowest with this diet.

Stearic acid in milk has 2 main origins: intake and complete ruminal biohydrogenation of 18-unsaturated

Table 4. Effect of diet and experimental week on the proportions of fatty acids in milk fat (% of total fatty acid methyl esters; n = 10)

Item ¹	Experimental diet						P-value			
	Control	Lentil straw	<i>Atriplex</i> leaves	Olive leaves	Olive cake	Tomato pomace	SEM	Diet	Week	Diet × week
16:0	33.2 ^a	31.7 ^b	32.6 ^{ab}	34.1 ^a	29.8 ^c	28.1 ^d	0.20	<0.001	0.772	0.219
18:0	7.2 ^c	6.7 ^{cd}	6.2 ^d	4.7 ^e	10.0 ^b	12.4 ^a	0.182	<0.001	0.032	0.005
18:1 <i>c9</i>	11.8 ^b	12.2 ^b	12.3 ^b	10.3 ^c	20.4 ^a	21.3 ^a	0.30	<0.001	<0.001	0.066
Total 18:1 <i>cis</i> ²	12.7 ^b	13.4 ^b	13.3 ^b	11.3 ^c	21.7 ^a	23.0 ^a	0.317	<0.001	<0.001	0.086
18:1 <i>t10</i>	0.415 ^b	1.214 ^a	0.521 ^b	1.008 ^a	0.483 ^b	0.568 ^b	0.0335	<0.001	<0.001	<0.001
18:1 <i>t11</i>	0.854 ^{bc}	1.060 ^{ab}	0.903 ^{bc}	1.259 ^a	0.690 ^c	1.073 ^{ab}	0.0328	<0.001	<0.001	<0.001
Total 18:1 <i>trans</i> ²	1.98 ^b	3.15 ^a	2.12 ^b	3.08 ^a	2.33 ^b	2.92 ^a	0.066	<0.001	<0.001	<0.001
18:2 <i>c9,c12</i>	3.16 ^a	3.43 ^a	3.25 ^a	3.34 ^a	2.53 ^c	2.83 ^b	0.038	<0.001	<0.001	0.277
18:2 <i>c9,t11</i>	0.349 ^c	0.507 ^{ab}	0.429 ^{bc}	0.580 ^a	0.364 ^c	0.616 ^a	0.0131	<0.001	0.011	<0.001
18:2 <i>c9,t12</i>	0.066 ^b	0.075 ^b	0.066 ^b	0.068 ^b	0.070 ^b	0.117 ^a	0.0017	<0.001	<0.001	0.357
18:2 <i>t11,c15</i> + <i>t9,c12</i>	0.072 ^d	0.084 ^{bcd}	0.089 ^{bc}	0.119 ^a	0.077 ^{cd}	0.082 ^{cd}	0.0018	<0.001	0.315	0.146
Total CLA ²	0.431 ^c	0.603 ^{ab}	0.515 ^{bc}	0.671 ^a	0.443 ^c	0.686 ^a	0.0134	<0.001	0.005	<0.001
18:3 <i>c9,c12,c15</i>	0.141 ^c	0.253 ^b	0.280 ^b	0.419 ^a	0.133 ^c	0.165 ^c	0.0071	<0.001	<0.001	<0.001
20:5 <i>c5,c8,c11,c14,c17</i>	0.018 ^c	0.028 ^b	0.030 ^b	0.037 ^a	0.018 ^c	0.020 ^c	0.0009	<0.001	<0.001	0.001
22:5 <i>c7,c10,c13,c16,c19</i>	0.048 ^{cd}	0.055 ^{bc}	0.063 ^{ab}	0.068 ^a	0.049 ^{cd}	0.045 ^d	0.0013	<0.001	<0.001	0.033
22:6 <i>c4,c7,c10,c13,c16,c19</i>	0.017 ^b	0.019 ^{ab}	0.021 ^{ab}	0.022 ^{ab}	0.027 ^a	0.022 ^{ab}	0.0008	0.035	0.030	0.591
Total n-3	0.469 ^c	0.602 ^b	0.638 ^b	0.811 ^a	0.437 ^c	0.454 ^c	0.0100	<0.001	<0.001	0.001
Total n-6	4.43 ^{ab}	4.89 ^a	4.53 ^{ab}	4.72 ^a	3.72 ^b	4.76 ^a	0.052	<0.001	<0.001	<0.001
n-6:n-3 ratio ³	9.57 ^a	8.12 ^b	7.10 ^c	5.82 ^d	8.51 ^b	10.48 ^a	0.163	<0.001	<0.001	<0.001
SCFA	17.4 ^b	18.1 ^{ab}	18.0 ^{ab}	18.8 ^a	14.0 ^c	11.9 ^d	0.19	<0.001	<0.001	0.109
MCFA	53.8 ^a	51.3 ^b	53.3 ^a	54.8 ^a	46.0 ^c	42.8 ^d	0.34	<0.001	0.649	0.059
SFA	77.9 ^a	75.5 ^b	76.9 ^{ab}	77.3 ^{ab}	69.6 ^c	66.2 ^d	0.35	<0.001	0.055	0.001
MUFA	16.9 ^d	18.7 ^c	17.5 ^{cd}	16.7 ^d	26.0 ^b	28.4 ^a	0.32	<0.001	0.242	0.180
PUFA	5.13 ^c	5.76 ^{ab}	5.50 ^{bc}	5.94 ^a	4.44 ^d	5.41 ^c	0.053	<0.001	<0.001	0.264

^{a-c}Mean values within the same row sharing no common superscript are significantly different ($P < 0.05$).

¹*c* = *cis*; *t* = *trans*; CLA = conjugated linoleic acids; SCFA = short-chain fatty acids, 4:0 to 11:0; MCFA = medium-chain fatty acids, 12:0 to 16:1; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

²Total 18:1 *cis* = sum of 18:1 *c9* and 18:1 *c11* to *c14*; total 18:1 *trans* = sum of 18:1 *t4* to *t12*; total CLA = sum of 18:2 *c9,t11*, 18:2 *c9,c11*, and 18:2 *t9, t11*.

³Unit without dimension.

FA (Chilliard et al., 2007; Gómez-Cortés et al., 2008). Accordingly, its excretion with milk was far higher than the amount consumed. This FA was especially enriched in the milk fat of diets with olive cake (rich in dietary 18:1 *c9*) and tomato pomace (rich in dietary 18:2 *c9,c12*). For olive cake, this was different than the results of Chiofalo et al. (2004). According to Gómez-Cortés et al. (2008), the biohydrogenation of 18:1 *c9* would generate 18:0, together with a variety of 18:1 *trans*, with the exception of 18:1 *t11*, which is consistent with the present observations of a low transfer rate of C18:1 *c9* from diet to milk when feeding olive cake.

n-3 Fatty Acids. All tested forages increased proportions of 18:3 *c9,c12,c15* and, less clearly, 20:5 *c5,c8,c11,c14,c17*, 22:5 *c7,c10,c13,c16,c19* and 22:6 *c4,c7,c10,c13,c16,c19* in milk fat compared with the control and diets with olive cake and tomato pomace. This indicates that the increase was a consequence of decreased biohydrogenation of 18:3 *c9,c12,c15* and not that of extra intake of this FA or EE. When evaluated across all diets, the proportion of dietary 18:3 *c9,c12,c15* recovered in milk was clearly decreasing with increasing intake, as has been found before (e.g., Kälber et al., 2011). Its content in milk fat even decreased when 6%

Table 5. Effect of diet and experimental week on the recovery rate of selected fatty acids (FA; g of FA excreted in milk/g of FA intake; n = 10)

Item ¹	Experimental diet						P-value			
	Control	Lentil straw	<i>Atriplex</i> leaves	Olive leaves	Olive cake	Tomato pomace	SEM	Diet	Week	Diet × week
18:3 <i>c9,c12,c15</i>	0.146 ^a	0.084 ^c	0.128 ^{ab}	0.103 ^{bc}	0.109 ^{bc}	0.039 ^d	0.0035	<0.001	<0.001	0.030
18:2 <i>c9,c12</i>	0.097 ^a	0.072 ^b	0.103 ^a	0.094 ^a	0.089 ^{ab}	0.031 ^c	0.0024	<0.001	<0.001	0.625
18:1 <i>c9</i>	0.935 ^a	0.644 ^b	0.953 ^a	0.688 ^b	0.411 ^c	0.641 ^b	0.0206	<0.001	<0.001	0.463
18:0	4.53 ^a	2.97 ^b	4.69 ^a	3.10 ^b	2.42 ^{bc}	1.77 ^c	0.107	<0.001	0.001	0.190

^{a-d}Mean values within the same row sharing no common superscript are significantly different ($P < 0.05$).

¹*c* = *cis*.

olive oil was added to the diet (Gómez-Cortés et al., 2008). As 18:3 *c9,c12,c15* is the precursor of the very long-chain n-3 FA (Chilliard et al., 2007), its increase in milk fat with the olive leaf diet was associated with these FA. Partially inhibited ruminal biohydrogenation due to phenols was described before for condensed tannins (Khiaosa-Ard et al., 2009), but this does not explain the present finding, as this inhibition did not encompass 18:3 *c9,c12,c15* biohydrogenation itself.

Biohydrogenation Intermediates. The ruminal biohydrogenation of 18:2 *c9,c12* and 18:3 *c9,c12,c15* and the isomerization of 18:1 *c9* result in a series of intermediates, whereof 2 specific isomers, 18:1 *t10* and 18:1 *t11*, are of particular interest (Gómez-Cortés et al., 2008; Khiaosa-Ard et al., 2010). The 18:1 *t11* isomer is formed from both 18:2 *c9,c12* and 18:3 *c9,c12,c15*, whereas 18:1 *t10* originates from 18:2 *c9,c12* only (Chilliard et al., 2007). Gómez-Cortés et al. (2008) pointed out that 18:1 *t10* is primarily produced from the isomerization of 18:1 *c9*.

When feeding the olive leaf diet, containing 9.3% 18:3 *c9,c12,c15* of total dietary FAME, this FA was probably the main substrate for biohydrogenation, as this resulted in an increase in 18:1 *t11*, consistent with results of Tsiplakou and Zervas (2008), and a clear increase in 18:2 *t11,c15* as well (Chilliard et al., 2007). In addition, 18:2 *c9,t11* was elevated. This CLA isomer, although being an intermediate in ruminal biohydrogenation of 18:2 *c9,c12*, is mainly synthesized by Δ^9 -desaturation of 18:1 *t11* in the mammary gland (Griinari et al., 2000; Chilliard et al., 2007). One source of condensed tannins tested by Khiaosa-Ard et al. (2009) was found to inhibit one important step in biohydrogenation and, therefore, resulted in 18:1 *t11* accumulation and 18:0 depletion. This was similar to the present findings. Among the test feeds, olive leaves were highest in total phenols and total tannins, though not in condensed tannins, suggesting that a comparable mechanism influenced microbial biohydrogenation. Still a proportionately higher increase occurred in 18:1 *t10* than in 18:1 *t11* with olive leaves, although its main precursor, 18:2 *c9,c12*, was low in the dietary lipids. As the olive leaf diet in our study was higher in non-NDF carbohydrates than the control diet, a certain decrease in rumen fluid pH could have occurred, which is known to facilitate 18:1 *t10* formation (Chilliard et al., 2007). This may have been caused by a shift from *Butyrivibrio fibrisolvens*, which isomerizes the *c12* bond to *t11*, to *Megasphaera elsdenii*, which isomerizes the *c9* bond to *t10* (Kramer et al., 2004).

In the case of tomato pomace and lentil straw, where the most abundant FA in the diet was 18:2 *c9,c12* (36.5 and 35.1% of total FAME in the diet, respectively), an increase in 18:1 *t11* was also found. According to

Bauman et al. (1999), high levels of 18:2 *c9,c12* would inhibit further biohydrogenation of the intermediate 18:1 *t11*, resulting in its accumulation as the major end product instead of 18:0. With lentil straw, 18:1 *t10* accumulation in milk fat was the highest of all diets but, different from the olive leaves, probably because enough substrate was available (18:2 *c9,c12*).

CONCLUSIONS

Several forages and agro-industrial byproducts, typical for dry areas, offer the opportunity to improve milk fat properties. An added value may be obtained by enhancing the proportion of FA with potential health benefits, namely 18:1 *c9* (with olive cake and tomato pomace), n-3 PUFA, and 18:2 *c9,t11* (with olive leaves and lentil straw), as well as the antioxidative stability of the milk (with saltbush leaves). As effects and modes of action largely differed, combinations of these feeds might be complementary and, thus, may improve milk fat properties in several aspects at once.

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