Dietary linseed oil increases *trans*-10,*cis*-15 18:2 in caprine milk fat

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ABSTRACT

Trans-10,*cis*-15 18:2 has been recently detected and characterized in digestive contents and meat and adipose tissue of ruminants, but its presence in milk and dairy products is hardly known. The aim of this study was to quantify *trans*-10,*cis*-15 18:2 in milk fat, better understand its metabolic origin, and help to elucidate the mechanisms of rumen biohydrogenation when the diet composition might affect ruminal environment. To address these objectives, 16 dairy goats were allocated to 2 simultaneous experiments (2 groups of goats and 2 treatments in each experiment). Experimental treatments consisted of basal diets with the same forage-to-concentrate ratio (33/67) and 2 starch-to-nonforage neutral detergent fiber (NDF) ratios (0.8 and 3.1), which were supplemented or not with 30 g/d of linseed oil for 25 d in a crossover design. *Trans*-10,*cis*-15 18:2 contents in milk fat were determined by gas chromatography fitted with an extremely polar capillary column (SLB-IL111). Levels of *trans*-10,*cis*-15 18:2 in individual milk fat samples ranged from 0 to 0.2% of total fatty acids, and its content in milk fat increased 8 fold due to linseed oil supplementation, substantiating the predominant role of α -linolenic acid in its formation. The *trans*-10,*cis*-15 18:2 levels in milk fat were similar in both experiments, despite the fact starch-to-nonforage NDF ratio of their respective basal diets greatly differed. In conclusion, *trans*-10,*cis*-15 18:2 was clearly related to linseed oil supplementation, and its increase in milk fat was comparable when the basal diets were rich in either nonforage NDF or starch.

Key words: milk fat, fatty acid, linseed oil, α -linolenic acid

INTRODUCTION

Milk fatty acid (FA) profile depends upon digestive processes and mammary gland lipid metabolism. Partial biohydrogenation and isomerization of PUFA by rumen bacteria generate a multiplicity of 18-carbon FA with different degree of unsaturation as well as positional and geometrical configurations, which are not completely characterized. The main route of α -linolenic acid biohydrogenation in the rumen first involves the isomerization of the *cis*-12 double bond to *trans*-11 (Figure 1). The obtained *cis*-9,*trans*-11,*cis*-15 18:3 is then hydrogenated to *trans*-11,*cis*-15 18:2, and finally to *trans*-11 18:1 (Harfoot and Hazlewood, 1997). Griinari and Bauman (1999) hypothesized that an altered rumen environment induced by feeding low-fiber diets could cause a shift in the biohydrogenation pathways of α -linolenic acid, favoring the isomerization of the *cis*-9 double bond to *trans*-10. In this way, *trans*-10 18:1 could originate not only from *cis*-9,*cis*-12 18:2 via *trans*-10,*cis*-12 18:2, but also from α -linolenic acid via *trans*-10,*cis*-12,*cis*-15 18:3 and *trans*-10,*cis*-15 18:2 (Figure 1).

Alves and Bessa (2014) first identified and characterized *trans*-10,*cis*-15 18:2 in digestive contents and meat from lambs fed a diet supplemented with linseed oil. More recently, Honkanen et al. (2016) found *trans*-10,*cis*-15 18:2, among other PUFA, as a result of α -linolenic acid metabolism during incubations with strained bovine rumen contents. However, evidence of the presence of that 18:2 isomer in milk and dairy products and quantification of its response to diet supplementation with α -linolenic acid sources is scant (De la Fuente et al., 2015; Manso et al., 2016).

In the present study, our first objective was to take advantage of the selectivity of the new ionic liquid capillary column of extreme polarity (SLB-IL111; Supelco, Bellefonte, PA) to confirm and quantify the presence of *trans*-10,*cis*-15 18:2 in milk fat. Second, to explore its metabolic origin, rations that differed in starch-to-nonforage NDF ratio and were supplemented or not with linseed oil were assayed in dairy goats.

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Figure 1. Pathways of ruminal biohydrogenation of α -linolenic acid postulated by Harfoot and Hazlewood (1997), Grinari and Bauman (1999), and Kishino et al. (2009).

MATERIALS AND METHODS

The present research was carried out at the University of Córdoba facilities in accordance with the EU Directive 2010/63/EU (EC, 2010) for animal studies. Sixteen Malagueña goats (initially 118 ± 16 DIM and 52.9 ± 3.7 kg of BW) were allocated to 2 experiments (2 treatments each), conducted simultaneously, in a crossover design with 4 animals per treatment and 2 experimental periods of 25 d. The goats were selected from a commercial farm and blocked by BW and milk production (4 animals in each block). Within each block the animals were randomly assigned to the treatments so all the blocks were represented in all the treatments. During the study, the goats were placed in individual cages of 1.0×1.4 m with slatted floors and individual water and feeding troughs. In both experiments, the basal diet consisted of alfalfa hay and a pelleted concentrate fed separately, had the same forage-to-concentrate ratio (33:67), and was supplemented or not with 30 g/d of linseed oil (Table 1). In experiment 1, the concentrate consisted of soybean hulls, barley, maize, and soybean meal, whereas the concentrate of experiment 2 did not include soybean hulls, which were replaced by equal parts of barley and maize. As a result, the starch-to-nonforage NDF ratio of the diet was 0.8 in experiment 1 and raised to 3.1 in experiment 2. Thus, 2 treatments were tested in each experiment: a basal diet with high nonforage NDF content (**F**) and the same diet plus linseed oil (**F+LO**) in experiment 1, and a basal diet with high starch content (**S**) and the same diet plus linseed oil (**S+LO**) in experiment 2. The diet was offered in 2 equal meals at 0930 and 1730 h. Voluntary

feed intake was recorded daily for each animal and was calculated as the difference between feed offered andorts. Water was provided ad libitum. The goats were machine milked and stripped out by hand once a day at 0830 h. Individual milk samples were collected on the last day of each experimental period and stored at -20°C until analysis.

Additionally, to collect further information on the *trans*-10,*cis*-15 18:2 metabolic origin, bulk milk samples (100 animals per tank) from a previous trial were re-analyzed (Gómez-Cortés et al., 2009). In Gómez-Cortés et al. (2009), 300 Manchega ewes receiving a 60:40 forage:concentrate diet were divided into 3 groups. One group was supplemented with 6% (DM basis) of extruded linseed (**LEL**), other group was supplemented with 12% (DM basis) of extruded linseed (**HEL**), and the last group (**CON**) was fed without linseed supplementation. Milk samples were collected at 0, 7, 14, 28, 45, and 60 d following supplementation. Housing, management, and diet composition are described in detail in Gómez-Cortés et al. (2009).

Milk fat extraction and derivatization of FA to FAME were carried out according to Bodas et al. (2010). Gas chromatography with 2 different columns was used to determine the content of selected FAME in accordance with the method proposed by De la Fuente et al. (2015). An Agilent model 6890 N Network Gas Chromatograph (Palo Alto, CA) equipped with auto-injector and flame ionization detector, fitted with a CP-Sil 88 fused silica capillary column (100 m $\times$ 0.25 mm i.d., Varian, Mid-delburg, the Netherlands) as well as an Agilent gas chromatograph, model 7820A GC System equipped with auto-injector and flame ionization detector, fitted with a SLB-IL111 capillary column (100 m $\times$ 0.25 mm i.d., Supelco) were used. Fatty acids were identified based on retention time and elution order as described by Alves and Bessa (2014).

We used SAS University Edition 3.4 (SAS Institute, Cary, NC) in the statistical analyses. Milk fat composition data from each experiment were analyzed with the MIXED procedure, according to the model:

$$Y_{ijklm} = \mu + T_i + P_j + S_k + G_l(S)_k + e_{ijklm},$$

where Y_{ijklm} = dependent variable, μ = overall mean, T_i = fixed effect of treatment ($i = 1$ to 2), P_j = fixed effect of period ($j = 1$ to 2), S_k = fixed effect of sequence ($k = 1$ to 2), $G_l(S)_k$ = random effect of animal ($l = 1$ to 4) nested within sequence k , e_{ijklm} = random residual. The CORR procedure was used to investigate the relationship between selected FA. Significant differences were declared at $P < 0.05$.

Table 1. Ingredients and chemical composition of the diets assayed

Item	Experiment 1		Experiment 2	
	F ¹	F+LO ¹	S ¹	S+LO ¹
Diet (g/d)				
Alfalfa hay	600	600	600	600
Pelleted concentrate ²	1,200	1,200	1,200	1,200
Linseed oil ³	—	30	—	30
Chemical composition				
DM (%)	89.5	90.0	89.2	89.7
Ash (% DM)	7.3	7.2	7.0	6.8
CP (% DM)	18.1	18.0	18.2	17.8
NDF (% DM)	40.7	40.4	27.5	27.7
Nonforage NDF (% DM)	23.5	23.2	10.3	10.5
Starch (% DM)	19.7	18.3	33.6	32.1
Fat by acid hydrolysis (% DM)	2.1	3.9	2.9	4.4
Fatty acids supplied (g/d)				
C16:0	4.6	6.3	5.0	6.7
C18:0	1.0	2.2	0.8	2.0
<i>cis</i> -9 C18:1	5.3	10.6	5.5	10.8
<i>cis</i> -9, <i>cis</i> -12 C18:2	13.7	18.4	14.3	19.0
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 C18:3	3.8	17.8	3.4	17.4

¹F = high nonforage NDF; F+LO = high nonforage NDF plus linseed oil; S = high starch; S+LO = high starch plus linseed oil.

²Composition (g/kg, as fed): soybean hulls, 356.0; maize, 178.0; barley, 178.0; and soybean meal, 250.0, in the F concentrate; and maize, 356.0; barley, 356.0; and soybean meal, 250.0, in the S concentrate. Both concentrates included (g/kg, as fed): vitamin and mineral premix (Trouwmix M-3020, Trouw Nutrition, Madrid, Spain), 30.0; binder (Exal; Tolsa SA, Madrid, Spain), 7.0; and antioxidant (Luctanox; Lucta SA, Barcelona, Spain), 1.0.

³The oil was included in the respective concentrate, during mixing before pelleting.

RESULTS AND DISCUSSION

Feed intake, milk yield, fat content, and protein content were in the ranges $1,645 \pm 168$ to $1,722 \pm 117$ g/d, $1,227 \pm 376$ to $1,552 \pm 592$ g/d, 3.83 ± 0.87 to $4.89 \pm 0.41\%$, and 3.49 ± 0.62 to $3.61 \pm 0.13\%$, respectively. Figure 2 depicts partial GC chromatograms showing the 18:2 region of milk FAME from goats fed the different diets (F, F+LO, S, and S+LO). In contrast to CP-Sil 88 column analysis, where *trans*-11,*cis*-15 and *trans*-10,*cis*-15 18:2 isomers co-eluted in the same peak, the new ionic liquid stationary phase of extreme polarity (SLB-IL111 capillary column) eluted separately both FA, thus permitting an easy quantification. In both experiments, *trans*-10,*cis*-15 18:2 levels were 8-fold higher in the milk from the linseed oil supplemented treatments compared with their respective controls (Table 2, Figure 2). It is noteworthy that the observed increase occurred regardless of the great difference in the starch to nonforage NDF of the basal diets between experiments. Most of the individual goat milk fat samples contained *trans*-10,*cis*-15 18:2, but in low or very low amounts. The highest content did not exceed 0.2% of total FA. Alves and Bessa (2014) first detected FA in digestive contents and meat from lambs, and Turner et al. (2015) determined its presence in bovine adipose tissue, but no quantitative information was given. Bravo-

Lamas et al. (2016) recently reported the contents of *trans*-10,*cis*-15 18:2 in lamb subcutaneous fat, providing values in the same range as those observed in the milk fat of the current study.

Table 2 shows the effects of the experimental diets fed to the goats on the milk fat contents of selected FA that are related to the metabolism of α -linolenic and linoleic acids in the rumen. Fatty acids from established ruminal biohydrogenation pathways of α -linolenic acid, such as *cis*-9,*trans*-11,*cis*-15 18:3 and *trans*-11,*cis*-15 18:2, significantly increased in the F+LO and S+LO treatments compared with their respective controls. In contrast, the observed contents were similar between the analogous treatments of both experiments (F vs. S and F+LO vs. S+LO). *Trans*-10,*cis*-15 18:2 followed the same pattern of the ruminal biohydrogenation products of α -linolenic acid, which would indicate that α -linolenic acid supplied by linseed oil was indispensable to generate *trans*-10,*cis*-15 18:2 in the rumen and to increase its contents in milk fat. Furthermore, *trans*-10,*cis*-15 18:2 was well correlated with α -linolenic acid in milk fats ($r = 0.79$; $P < 0.001$), supporting the idea that both PUFA are closely linked. Our results are in line with those of Honkanen et al. (2016), who related *trans*-10,*cis*-15 18:2 with α -linolenic acid metabolism by rumen microbes in vitro, as well as those of Alves and Bessa (2014) and Turner et al. (2015), who found

Table 2. Contents (g/100 g of FAME) of selected fatty acids in the milk fat of dairy goats fed 2 basal diets, in separate experiments, supplemented or not with linseed oil

Item	Experiment 1 ¹				Experiment 2 ¹			
	F	F+LO	SEM	P-value	S	S+LO	SEM	P-value
<i>trans</i> -10 18:1	0.32	0.46	0.035	<0.05	0.58	0.55	0.090	0.89
<i>trans</i> -11 18:1	1.15	3.26	0.299	<0.001	1.87	3.25	0.223	<0.001
<i>trans</i> -10, <i>cis</i> -15 18:2	0.01	0.08	0.012	<0.05	0.01	0.08	0.012	<0.01
<i>trans</i> -11, <i>cis</i> -15 18:2	0.05	0.67	0.084	<0.001	0.09	0.55	0.065	<0.001
<i>trans</i> -10, <i>cis</i> -12 18:2	0.01	0.01	0.001	0.35	0.01	0.01	0.001	0.92
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15 18:3	0.48	0.97	0.080	<0.001	0.35	0.66	0.046	<0.001
<i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15 18:3	0.04	0.08	0.008	<0.05	0.04	0.06	0.004	<0.01
<i>trans</i> -10 18:1/ <i>trans</i> -11 18:1	0.29	0.14	0.022	<0.001	0.29	0.17	0.031	0.06
<i>trans</i> -10 18:1/ <i>trans</i> -10, <i>cis</i> -12 18:2	142	207	28.1	0.27	177	178	22.6	0.97
<i>trans</i> -10 18:1/ <i>trans</i> -10, <i>cis</i> -15 18:2	29	7	3.0	<0.05	78	8	13.5	<0.001

¹F = high nonforage NDF; F+LO = high nonforage NDF plus linseed oil; S = high starch; S+LO = high starch plus linseed oil.

trans-10,*cis*-15 18:2 in ruminant tissues from animals fed linseed oil and linseed supplemented diets, respectively.

On the other hand, the immediate precursor of *trans*-10,*cis*-15 18:2 in milk would be somewhat uncertain. We did not detect its putative precursor *trans*-10,*cis*-12,*cis*-15 18:3 in any of the samples analyzed, and neither did Alves and Bessa (2014) in their study. However, Honkanen et al. (2016) detected *trans*-10,*cis*-12,*cis*-15

18:3 positional isomers in incubations of α-linolenic acid with strained bovine rumen contents but, at such low amounts, that no conclusions could be drawn about the possible mechanisms involved. Kishino et al. (2009) found *trans*-10,*cis*-15 18:2 in cells of *Lactobacillus plantarum* after incubation with α-linolenic acid. Those authors suggested a metabolic pathway in which *trans*-10,*cis*-15 18:2 is formed from α-linolenic acid via conjugated *cis*-9,*trans*-11,*cis*-15 18:3 and *trans*-9,*trans*-

Figure 2. Partial GC chromatograms of the 18:2 region using an extremely polar capillary column (SLB-IL111, Supelco, Bellefonte, PA). The analyzed milk samples are from goats fed high-starch (top) or high-fiber diets (bottom) with (---) or without (—) linseed oil supplementation (30 g/d).

11,*cis*-15 18:3, without the production of *trans*-10 18:1 (Figure 1). In the study of Honkanen et al. (2016), both 18:3 isomers, mainly *cis*-9,*trans*-11,*cis*-15, were detected during the first hour of incubation and their amounts declined thereafter. In the present study, *cis*-9,*trans*-11,*cis*-15 18:3 was significantly increased in the milk fat from goats fed the linseed oil supplemented diets (Table 2), but *trans*-9,*trans*-11,*cis*-15 18:3 was not detected. The lack of this intermediate in milk could be justified by its low production in the rumen, as *L. plantarum* is not recognized as being a predominant microorganism in the ruminal ecosystem (Lourenço et al., 2010).

It is noteworthy that linseed oil supplementation only raised the content of *trans*-10 18:1 in experiment 1 (high-fiber diets), but the value reached in the F+LO treatment was not as high as those observed in any of the treatments of experiment 2 (high-starch diets; Table 2). On the contrary, the observed increase of *trans*-10,*cis*-15 18:2 due to linseed oil in the diet was similar in both experiments. These results would indicate that a high starch intake favored the production of *trans*-10 18:1 in the rumen, but had little relevance to generate *trans*-10,*cis*-15 18:2. In this regard, it has been stated that a high proportion of starch in the diet contributes to modify the ruminal biohydrogenation pathways, with the largest *trans*-10 18:1 shift being associated with the abundance of linoleic acid, but not α -linolenic acid, in the rations (Zened et al., 2011, 2013). Furthermore, the *trans*-10 18:1-to-*trans*-10,*cis*-15 18:2 ratio in milk fat significantly decreased when linseed oil was included in both experiments (Table 2), thus indicating that an increase of the substrate, *trans*-10,*cis*-15 18:2, did not cause a parallel rising in milk *trans*-10 18:1 levels and that the formation of *trans*-10 18:1 from *trans*-10,*cis*-15 18:2 in the rumen would not be remarkable. In comparison, the *trans*-10 18:1 to *trans*-10,*cis*-12 18:2 ratio in milk fat was not modified in the F+LO and S+LO treatments compared with the F and S treatments, respectively, which would reveal that their metabolic relationship could be relatively independent of the inclusion of linseed oil in goat diets (Martínez Marín et al., 2015).

To confirm the role of α -linolenic acid in the metabolic origin of *trans*-10,*cis*-15 18:2, sheep bulk milk fats, sampled over time in a previous trial (Gómez-Cortés et al., 2009), were reanalyzed by GC using the SLB-IL111 column. The obtained data did not allow us to investigate statistical differences, but the results are of interest from a qualitative point of view. The LEL and HEL diets sharply increased the *trans*-10,*cis*-15 18:2 levels in milk fat during the first week of supplementation (from 0.01% of total FAME at d 0 in both treatments to 0.05 and 0.07% of total FAME in the LEL and HEL treatments, respectively) and did not decrease

from that level thereafter. The highest level (0.10% of total FAME) was reached at the end of the monitoring period in the HEL treatment. *Trans*-10,*cis*-15 18:2 contents in milk fat showed a high correlation with those of α -linolenic acid ($r = 0.99$, $P < 0.001$), indicating once more that both FA are closely related. Moreover, the *trans*-10 18:1-to-*trans*-10,*cis*-15 18:2 ratio in milk fat remained relatively unchanged during the experimental period in the CON treatment (44 ± 11), whereas it was substantially lower from the first week onwards in the LEL and HEL treatments (9 ± 1 and 6 ± 1 vs. initial values of 32 and 36, respectively). These observations indicate that dietary linseed had a considerable influence on the presence of *trans*-10,*cis*-15 18:2 in the milk fat of the ewes, but did not entail an increase of *trans*-10 18:1, in agreement with previously mentioned results. Therefore, under the experimental conditions assayed, *trans*-10 18:1 would not be formed from *trans*-10,*cis*-15 18:2 in goats and ewes.

Overall, this research confirms the predominant role of α -linolenic acid as precursor of *trans*-10,*cis*-15 18:2 in milk fat and dismisses *trans*-10,*cis*-15 18:2 as precursor of *trans*-10 18:1, at least under the conditions assayed. The present investigation did not allow us to elucidate the routes explaining the conversion of α -linolenic acid to *trans*-10,*cis*-15 18:2 and further research needs to be done, as minor intermediates not present in milk or still unknown may play a relevant role.

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