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Odd- and branched-chain fatty acids in goat milk as indicators of the diet composition

Alfonso Cívico^a, Nieves Núñez Sánchez^a (b), Pilar Gómez-Cortés^b, Miguel Angel de la Fuente^b, Francisco Peña Blanco^a, Manuela Juárez^b, Achille Schiavone^c (b) and Andrés Luis Martínez Marín^a (b)

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ABSTRACT

This work aimed to determine if the milk fat contents of odd- and branched-chain fatty acids (OBCFA) could be linked to the diet composition in goats, by canonical discriminant analysis (CDA). The OBCFA contents of 32 milk fat samples from two feeding trials (two treatments per trial) were used. In both experiments, the goats were fed a diet, with the same forage/concentrate ratio and nutritive value, which was supplemented or not with 30 g/d of linseed oil. The starch/non-forage NDF ratio of the diet was 3.1 in the first experiment and was lowered to 0.8 in the second experiment by replacing part of the cereals in the concentrate with soybean hulls. Milk fat composition analyses were grouped into four classes in the CDA. Stepwise backward selection identified C13:0 iso, C14:0 iso, C17:0 anteiso, C18:0 iso and C17:0 + cis-9 C17:1 as valid predictors. The first two discriminant functions (DF) explained 82.2 and 13.9% of total variance. DF1 and DF2 differentiated milk samples by the non-forage NDF content and linseed oil supplementation of the diet, respectively. C14:0 iso and C17:0 anteiso were indicative of the diets with high non-forage NDF and starch contents, respectively. C17:0 + cis-9 C17:1 and both C18:0 iso and C13:0 iso contributed to identify the diets with and without added linseed oil, respectively. In conclusion, CDA allowed to identify which milk OBCFA were the best indicators of the starch/ non-forage NDF ratio and the presence of linseed oil in the diets fed to dairy goats.

Introduction

Odd- and branched-chain (iso and anteiso) fatty acids (OBCFA) in milk fat largely derive from rumen bacteria, which in turn show large differences in their OBCFA profile (Vlaeminck et al. 2006; Fievez et al. 2012). Cellulolytic bacteria contain more iso fatty acids (FA), whereas amylolytic bacteria are enriched in anteiso and linear odd-chain FA and show relatively low levels of iso FA (Vlaeminck et al. 2006; Fievez et al. 2012). Most of OBCFA are incorporated into milk fat without further modifications, but *de novo* synthesis and Δ -9 desaturation of C15:0 and C17:0, as well as elongation of C15:0 iso and C15:0 anteiso to render C17:0 iso and C17:0 anteiso may occur (Fievez et al. 2003; French et al. 2012; Vlaeminck et al. 2015). In ruminant milk fat, the contents of odd- and branched-chain FA may vary in the range of 2.0-3.1% and 1.4-2.4%, respectively (Devle et al. 2012). Regardless their low percentages in milk fat, OBCFA are of great interest for their potential role as non-invasive biomarkers of rumen function, since variations in milk OBCFA could reflect changes of rumen bacterial populations induced by diet composition (Vlaeminck et al. 2006; Fievez et al. 2012). In addition, OBCFA have potential health benefits in humans (Cai et al. 2013; Jenkins et al. 2015).

Alonso et al. (1999) identified many OBCFA in goat milks from different commercial farms. Later studies have shown that OBCFA contents in goat milk fat depend on diet characteristics such as the forage/concentrate ratio (Serment et al. 2011), the plant oil supplementation (Bernard et al. 2009; Martínez Marín et al. 2011) and its interaction with forage level (Mele et al. 2008; Ollier et al. 2009), and the percentage of NDF physically effective (Li et al. 2014a). Moreover, the proportions of OBCFA in the rumen contents of goats

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are affected by the NDF physically effective/rumen degradable starch ratio of the diet (Li et al. 2014b). To the best of our knowledge there is no published research on the effects of the starch/non-forage NDF ratio of the diet and its interaction with linseed oil supplementation, at the same forage/concentrate ratio, on the OBCFA contents in goat milk fat.

Discriminant analysis has proven to be an effective method to test the ability of milk FA contents to predict the type of diet consumed by dairy goats (Martínez Marín et al. 2012), as well as to distinguish changes of the rumen fermentation pattern due to dietary manipulations in cows (Van Nespen et al. 2005; Colman et al. 2010). Thus, the aim of this paper was to investigate the relationship between the OBCFA contents in milk fat and the composition of diets fed to dairy goats, by canonical discriminant analysis.

Materials and methods

Fat composition of 32 milk samples from 16 Malagueña goats (initially 118±16 days in milk and 52.9 ± 3.7 kg of body weight) allocated to two feeding trials (unpublished study) were used. The study was carried out at the University of Córdoba facilities in accordance with the EU Directive 2010/63/EU for animal experiments. The feeding trials were conducted simultaneously in a cross-over design with four animals per treatment (control and supplemented) and two experimental periods of 25 days. The goats were selected from a commercial farm and blocked by body weight and milk production. Then, the blocks were randomly assigned to the treatments of each experiment. During the study, the goats were placed in individual cages of 1.0×1.4 m with a slatted floor and water and feeding troughs. In both experiments, the diet consisted of alfalfa hay as the sole forage and a pelleted concentrate, had the same forage/concentrate ratio, and was supplemented or not with 30 g/d of linseed oil (Table 1). In the first experiment, the concentrate consisted of barley, maize and soybean meal, whereas the concentrate of the second experiment included soybean hulls replacing equal amounts of barley and maize. The oil was added on the meal during mixing (horizontal mixer) prior to pelleting. The starch/non-forage NDF ratio of the diet was 3.1 in the first experiment and was lowered to 0.8 in the second experiment. Since the forage/concentrate ratio was the same in both experiments, most NDF in the diet of the second experiment was non-forage NDF. The diet was offered in 2 equal meals at 0930 and 1730 h. Water was provided ad libitum.

Table 1. Composition of the diets.

	Treatments ^a			
	S	SL	F	FL
Diet, g/d				
Alfalfa hay	600	600	600	600
Pelleted concentrate ^b	1200	1200	1200	1200
Linseed oil ^c	-	30	-	30
Chemical composition				
Dry matter (DM), %	89.2	89.7	89.5	90.0
Ash, % DM	7.0	6.8	7.3	7.2
Crude protein, % DM	18.2	17.8	18.1	18.0
Neutral detergent fibre (NDF), % DM	27.5	27.7	40.7	40.4
Non-forage NDF, % DM	10.3	10.5	23.5	23.2
Starch, % DM	33.6	32.1	19.7	18.3
Fat by acid hydrolysis, % DM	2.9	4.4	2.1	3.9
Fatty acids supplied, g/d				
C16:0	5.0	6.7	4.6	6.3
C18:0	0.8	2.0	1.0	2.2
<i>cis</i> -9 C18:1	5.5	10.8	5.3	10.6
cis-9, cis-12 C18:2	14.3	19.0	13.7	18.4
cis-9, cis-12, cis-15 C18:3	3.4	17.4	3.8	17.8

^aS: high starch; SL: high starch plus linseed oil; F: high non-forage NDF; FL: high non-forage NDF plus linseed oil.

^bComposition (g/kg, as fed): maize, 356.0; barley, 356.0; and soybean meal, 250.0, in the S concentrate; and maize, 178.0; barley, 178.0; soybean hulls, 356.0; and soybean meal, 250.0, in the F 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.

^cThe oil was included in the respective concentrate.

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. Milk fat was extracted as described by Luna et al. (2005), placed in amber vials, blanketed with a stream of nitrogen and stored at -20°C until analysis. Fatty acid methyl esters (FAME) were prepared by base-catalysed methanolysis of glycerides with KOH in methanol (ISO-IDF 2002). Gas chromatography with two different columns was used to determine the FAME profile 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 autoinjector, fitted with a FID on a CP-Sil 88 fused silica capillarv column $(100 \,\mathrm{m} \times 0.25 \,\mathrm{mm}$ i.d., Varian, Middelburg, The Netherlands), was used. Injector and detector temperature was 250 °C. He was the carrier gas, at an inlet pressure of 193.9 kPa and a split ratio of 1:100. Initial oven temperature was 45 °C. After 4 min, oven temperature was raised at 13 °C/min to 165 °C and held for 35 min, then increased to 215 °C at 4°C/min and maintained for 30 min. Another Agilent gas chromatograph, model 7820A GC System equipped with auto-injector and FID, was fitted with an SLB-IL111 capillary column ($100 \text{ m} \times 0.25 \text{ mm}$ i.d., Supelco). Injector and detector temperature was 250 °C. The column inlet pressure was set at 241 kPa, resulting in He gas flow rates of 0.8 mL/min.

Fatty acids	Treatments ^a					
	S	SL	F	FL		
Saturated	72.62 ± 2.96	68.06 ± 1.05	73.84 ± 2.00	68.28 ± 2.66		
Monounsaturated	22.81 ± 2.49	25.56 ± 1.14	21.32 ± 1.88	24.56 ± 2.32		
Polyunsaturated	4.54 ± 0.63	6.28 ± 0.66	4.76 ± 0.46	7.17±0.94		
Total C18:1 trans	3.61 ± 0.97	5.72 ± 0.78	2.40 ± 0.63	5.68 ± 0.94		
Total C18:2 conjugated	0.99 ± 0.16	1.44 ± 0.25	0.69 ± 0.14	1.58 ± 0.39		
Total omega-3	0.59 ± 0.09	1.10 ± 0.17	0.75 ± 0.13	1.56 ± 0.34		

Table 2. Contents (mean \pm standard deviation, g per 100 g of total fatty acid methyl esters) of the main groups of fatty acids in the milk fat of goats fed with different diets.

^aS: high starch; SL: high starch plus linseed oil; F: high non-forage NDF; FL: high non-forage NDF plus linseed oil.

Table 3. Odd- and branched-chain fatty acid contents (mean \pm standard deviation, g per 100 g of total fatty acid methyl esters) and univariate test of equality between group means of the diet classes used in the canonical discriminant analysis.

		Treatments ^a					
Fatty acids	S	SL	F	FL	p		
C13:0 iso	0.014 ± 0.007	0.013 ± 0.002	0.019 ± 0.004	0.013 ± 0.005	.046		
C13:0 anteiso	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	.192		
C14:0 iso	0.032 ± 0.006	0.035 ± 0.005	0.072 ± 0.006	0.071 ± 0.017	<.001		
C15:0 iso	0.139 ± 0.030	0.146 ± 0.030	0.171 ± 0.028	0.150 ± 0.049	.335		
C15:0 anteiso	0.223 ± 0.033	0.239 ± 0.048	0.304 ± 0.065	0.284 ± 0.084	.043		
C16:0 iso	0.145 ± 0.042	0.141 ± 0.051	0.186 ± 0.028	0.167 ± 0.034	.106		
C17:0 iso	0.232 ± 0.029	0.207 ± 0.029	0.271 ± 0.017	0.241 ± 0.048	.005		
C17:0 anteiso	0.260 ± 0.039	0.275 ± 0.055	0.294 ± 0.069	0.235 ± 0.066	.244		
C18:0 iso	0.043 ± 0.015	0.034 ± 0.004	0.050 ± 0.004	0.034 ± 0.003	.001		
C15:0	0.617 ± 0.054	0.754 ± 0.105	0.815 ± 0.079	0.760 ± 0.133	.003		
C17:0 + <i>cis</i> -9 C17:1	0.638 ± 0.112	0.677 ± 0.056	0.768 ± 0.035	0.652 ± 0.060	.005		

^aS: high starch; SL: high starch plus linseed oil; F: high non-forage NDF; FL: high non-forage NDF plus linseed oil.

Sample was injected at a split ratio of 1:100. Initial oven temperature was isothermal (168 $^{\circ}$ C), and after 45 min it was raised at 5 $^{\circ}$ C/min to 210 $^{\circ}$ C and held for 36.6 min. The use of SLB-IL111 column complemented the analysis of CP-Sil88 allowing the discrimination of some odd- and branched-chain FA.

The OBCFA contents in the milk fat samples were grouped into four classes, according to the four experimental treatments: diet with high starch content (S), diet with high starch content plus linseed oil (SL), diet with high non-forage NDF content (F), and diet with high non-forage NDF content plus linseed oil (FL).

Statistical analyses were performed with SAS University Edition 3.4 (SAS Institute, Cary, NC). The STEPDISC procedure was used to determine which OBCFA made a good contribution to the differentiation among the diet classes (S, SL, F and FL). Probability to enter and stay in the model was set at 0.10 and 0.20, respectively. The selected OBCFA were then used in the CANDISC procedure to perform a canonical discriminant analysis. The univariate ANOVA procedure, using the diet classes as fixed effect, and the CORR procedure were used when appropriate.

Results and discussion

Feed intake, milk yield, fat content and protein content were in the ranges $1645 \pm 168 - 1722 \pm 117 \text{ g/d}$,

 $1227 \pm 376 - 1552 \pm 592 \, \text{g/d},$ $3.83 \pm 0.87 - 4.89 \pm 0.41\%$ and $3.49 \pm 0.62 - 3.61 \pm 0.13\%$, respectively. The contents of the main groups of FA in milk fat are shown in Table 2. The range of total OBCFA content in goat milk fat was 2.4-3.0 g/100 g of total FAME (Table 3) which is in agreement with previous reports (Alonso et al. 1999; Serment et al. 2011; Devle et al. 2012). On the other hand, the groups of goats used in the experimental treatments of the present study were homogeneous, maintained under the same housing and management conditions, and the feeding trials were carried out simultaneously. Moreover, the diets had the same forage/concentrate ratio and supplied similar amounts of FA, as well as the amount of supplemented linseed oil was equal in both experiments (Table 1). Hence, differences in the milk OBCFA profile between animals, if any, should be related primarily to the effects of the starch/non-forage NDF ratio and/or the presence of linseed oil in the diets.

Five variables (four branched-chain FA and the sum of C17:0 and *cis*-9 C17:1) were selected as good predictors in the discriminant model (Table 4). Wilks' test indicated that the model was significant (p < .001) and explained 98.4% of the shared variance between variables. The first two canonical discriminant functions (DF) explained 82.2 and 13.9% of total variance (Figure 1). The squared Mahalanobis distances were significant in all the group comparisons (p < .001).

In DF1, the S and SL diets had negative centroid values while the F and FL diets had positive centroid values (Table 4). Therefore, this function clearly discriminated between the diets with high starch content and the diets with high non-forage NDF content (left and right sides of the plot in Figure 1, respectively). Standardised canonical discriminant coefficients (Table 4) showed that the FA with the greatest discriminating ability in DF1 were C14:0 *iso* in positive direction (i.e. opposite to the high starch diets) and C17:0 *anteiso* in negative direction (i.e. opposite to the high non-forage NDF diets) (Figure 1). We used

 Table 4. Canonical discriminant analysis results (1, 2 and 3 are the canonical functions).

	Standa c	rdised ca oefficient	nonical s	Canor	nical struc	ture
	1	2	3	1	2	3
C13:0 iso	0.461	1.066	0.025	0.089	0.196	0.511
C14:0 iso	1.594	-0.167	-0.135	0.588	-0.197	0.364
C17:0 anteiso	-1.459	-0.339	0.548	-0.012	0.102	0.482
C18:0 iso	0.832	1.791	-0.394	0.077	0.523	0.472
C17:0 + <i>cis</i> -9 C17:1	-0.594	-1.370	1.127	0.102	0.105	0.850
Eigenvalues	12.096	2.041	0.580			
Canonical correlation	0.924	0.671	0.367			
Variance explained, % Centroids ^a	82.19	13.87	3.94			
S	-2.638	1.655	-0.640			
SL	-3.815	-1.401	0.516			
F	3.153	0.970	0.882			
FL	3.299	-1.224	-0.758			

^aRations with high starch (S) or non-forage NDF (F) contents, either alone or in combination with linseed oil (SL and FL). univariate ANOVA to further confirm that the values of the C17:0 *anteiso* to C14:0 *iso* ratio in milk fat were higher (p < .05) in the S and SL than in the F and FL diets (Table 5).

Those results were in agreement with the positive and negative correlation of the NDF and starch contents of the diet, respectively, with the milk fat percentage of C14:0 iso that has been observed in dairy cows (Vlaeminck et al. 2006). In goats, increasing the ratio of NDF physically effective to rumen degradable starch in the diet linearly raised the proportion of C14:0 iso in the rumen content (Li et al. 2014b). Also, feeding goats a diet with high percentage of NDF physically effective increased the content of C14:0 iso in milk fat (Li et al. 2014a). Both Li et al. (2014a, 2014b) observed that the positive responses of the C14:0 iso proportion in their respective works were associated with the diets that showed a lower daily duration of rumen pH <5.8 or pH <5.6 and an abundance of cellulolytic bacteria relative to amylolytic bacteria. Cellulolytic bacteria contain predominantly iso FA in their membranes (Vlaeminck et al. 2006; Fievez et al. 2012) and are sensitive to low-rumen pH values as those observed when high starch diets are fed (Sun et al. 2010). Thereby, in the present work, the contribution of milk C14:0 iso to separate the high nonforage NDF diets from the high starch diets in DF1 (Table 4, Figure 1) would reflect a higher rumen pH in the F and FL treatments due to their lower starch/nonforage NDF ratio, which could favour the abundance



Figure 1. Canonical discriminant plot of the first 2 canonical functions (S, F, SL and FL: Observations corresponding to rations with high starch content (\blacksquare), high starch plus linseed oil (\blacklozenge), high NDF content (▲) and high NDF fibre plus linseed oil (\blacklozenge), respectively. Centroids are indicated by asterisks).

Table 5. Mean separation analysis for the ratios of the milk fatty acids with the highest discriminant abilities in the discriminant functions 1 and 2 (DF1 and DF2).

	Treatment				
	S	SL	F	FL	SEM
DF1: C17:0 anteiso/C14:0 iso	8.2ª	7.8 ^a	4.0 ^b	3.3 ^b	0.41
DF2: C17:0 + <i>cis</i> -9 C17:1/C13:0 <i>iso</i> + C18:0 <i>iso</i>	11.4 ^b	14.5 ^a	11.1 ^b	13.9 ^a	0.33
	,		(

S: high starch; SL: high starch plus linseed oil; F: high non-forage NDF; FL: high non-forage NDF plus linseed oil. ^{a,b}Within a row, means without a common superscript letter are significantly different by Tukey's test (p < .05).

of cellulolytic bacterial populations. The positive relationship between milk C14:0 *iso* and rumen pH is wellknown in dairy cows (Fievez et al. 2012; Baumann et al. 2016). Moreover, the contribution of milk C17:0 *anteiso* to identify the consumption of the high starch diets in DF1 (Table 4, Figure 1) would also suggest changes in the rumen bacterial populations or a bacterial response to rumen stress stimuli such as reduced rumen pH (Vlaeminck et al. 2006). Nevertheless, a more active elongation of C15:0 *anteiso* to C17:0 *anteiso* might also occur in the animals that consumed the S and SL diets (Vlaeminck et al. 2015).

In DF2, the S and F diets showed positive centroid values while the SL and FL diets showed negative centroid values (Table 4). Therefore, DF2 clearly discriminated between non-supplemented diets and those supplemented with linseed oil (upper and lower sides of the plot in Figure 1, respectively). Standardised canonical discriminant coefficients (Table 4) showed that the FA with the greatest discriminating ability in DF2 were C18:0 iso and C13:0 iso in positive direction (i.e. opposite to the linseed oil enriched diets) and C17:0 + cis-9 C17:1 in negative direction (i.e. opposite to the diets without linseed oil) (Figure 1). Univariate ANOVA results further confirmed that the values of the C17:0 + cis-9 C17:1 to C13:0 iso + C18:0 iso ratio in milk fat were higher (p < .05) in the SL and FL than in the S and F diets (Table 5).

The contribution of C18:0 *iso* and C13:0 *iso* to separate the non-supplemented diets from the diets supplemented with linseed oil in DF2 (Table 3, Figure 1) might reflect the absence of negative effects of the former diets on *de novo* FA synthesis in rumen bacteria (Demeyer et al. 1978). However, it does not preclude the possibility that reflected a direct inhibitory effect of the linseed oil supplied by the SL and FL diets on some rumen bacterial populations especially rich in *iso* FA as cellulolytic bacteria (Vlaeminck et al. 2006; Fievez et al. 2012). This statement would be supported by two different points. Firstly, cellulolytic bacteria are highly susceptible to unsaturated FA and particularly to α -linolenic acid (Yang et al. 2009), which was the main fatty acid supplied by linseed oil in the

present work (Table 1). Secondly, cellulolytic bacteria participate in the predominant rumen biohydrogenation (RBH) pathway, which results in the production of stearic acid from dietary unsaturated FA (Martin & Jenkins 2002). Hence, it might be expected that any negative effects of the linseed oil supplied by the SL and FL diets on cellulolytic bacteria would increase the RBH intermediates levels in milk fat whilst lowering its iso FA contents. In the current research, total trans C18:1 and total conjugated C18:2 contents in milk fat showed higher values in the linseed oil supplemented treatments (Table 2), and C18:0 iso was negatively correlated with both total trans C18:1 and total conjugated C18:2 (r = -.22 and -.19, respectively, p < .05). Also, Bernard et al. (2009) found that including linseed oil in dairy goat diets decreased the contents of most iso FA in milk fat with a concomitant increase in the proportions of total trans C18:1 and total conjugated C18:2.

Since amylolytic bacteria are relatively enriched in linear odd-chain FA (Vlaeminck et al. 2006; Fievez et al. 2012), the contribution of milk C17:0 + *cis*-9 C17:1 to identify the linseed oil supplemented diets in DF2 (Table 4, Figure 1) would suggest a more adverse effect of linseed oil on cellulolytic than on amylolytic bacterial populations, as observed in dairy cows by Yang et al. (2009). However, a more active elongation of C15:0 to C17:0 might also occur in the mammary gland under such diets (Vlaeminck et al. 2015).

Conclusions

Canonical discriminant analysis allowed to identify which milk OBCFA were the best indicators of the starch/non-forage NDF ratio and the presence of linseed oil in diets fed to dairy goats. It was found that a high C17:0 *anteiso* to C14:0 *iso* ratio in milk fat would be indicative of a high starch/non-forage NDF ratio in the diet as well as a high C17:0 + *cis*-9 C17:1 to C13:0 *iso* + C18:0 *iso* ratio in milk fat would be related to linseed oil supplementation. These results suggest that milk OBCFA are closely related to the diet composition in dairy goats and could be further investigated as

potential biomarkers of the diet effects on their rumen function.

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