Replacing cereals with dehydrated citrus pulp in a soybean oil supplemented diet increases vaccenic and rumenic acids in ewe milk

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ABSTRACT

This study evaluates the effect of the replacement of cereals by dried citrus pulp (DCP) in diets supplemented with 5% of soybean oil, on ewe milk yield and composition, including milk fatty acid (FA). Four Serra da Estrela multiparous ewes in the second month of lactation were used in a double 2 × 2 Latin square design. Ewes were individually penned and milked twice a day with an 8-h interval. Each experimental period included 14 d of diet adaptation followed by 5 d of measurements and sampling. The 2 diets included on dry matter basis 450 g/kg of corn silage and 550 g/kg of either a soybean oil-supplemented concentrate meal containing barley and maize (cereal) or dried citrus pulp (DCP; citrus). Feed was offered *ad libitum*, considering 10% of orts, and intake was measured daily. Milk yield was higher and dry matter intake tended to be higher with the citrus diet. Milk composition and technological properties for cheese production were not affected by treatments, except for lactose, which was lower with the citrus diet. Replacement of cereals by DCP resulted in a 3-percentage-point decrease of both 18:0 and *cis*-9-18:1 that were mostly compensated by the 4.19- and 1.68-percentage-point increases of *trans*-11-18:1 and *cis*-9,*trans*-11-18:2, respectively. The intake of C18 FA tended to increase with the citrus diet compared with the cereal diet, but the apparent transfer of 18:2n-6 and of 18:3n-3 did not differ between diets. The milk output of C18 FA increased with the citrus compared with the cereal diet, mostly due to the increase of *trans*-11-18:1 and *cis*-9,*trans*-11-18:2 because the daily milk output of 18:0, *trans*-10-18:1, *cis*-9-18:1, 18:2n-6 and 18:3n-3 did not differ between diets. Replacing cereals with

DCP in an oil-supplemented diet resulted in a selective increase of *trans*-11-18:1 and *cis*-9,*trans*-11-18:2 in milk, with no major effect on other biohydrogenation intermediates.

Key words: dairy ewe, dried citrus pulp, milk fatty acid, biohydrogenation intermediates, soybean oil

INTRODUCTION

Rumen lipid metabolism, particularly the biohydrogenation of PUFA, is a major determinant of the fatty acid (FA) profile of ruminant milk. Conjugated linoleic acid increase, particularly rumenic acid (*cis*-9,*trans*-11-18:2) and its precursor vaccenic acid (*trans*-11-18:1), is one of the main objectives for improving the nutritional value of milk fat (Pintus et al., 2013; Shingfield et al., 2013; Nudda et al., 2014). This goal can be achieved by supplementing ruminant diets with vegetable oils rich in PUFA (Shingfield et al., 2013) among other strategies. However, the nature of the carbohydrates present in these diets influence the rumen biohydrogenation pathways and thus the content of *trans*-11-18:1 and *cis*-9,*trans*-11-18:2 in products. Diets rich in starch and PUFA, particularly 18:2n-6, are frequently associated with changes in the dominant biohydrogenation pathways, markedly increasing the concentration of *trans*-10-18:1 in fat from ruminant products (*trans*-10 shift; Aldai et al., 2013; Bessa et al., 2015). In dairy cows, large increases in *trans*-10-18:1 are often accompanied by a decrease in *trans*-11-18:1 and *cis*-9,*trans*-11-18:2 and reduction of milk fat concentration (Shingfield and Griinari, 2007). Dairy ewes seem less susceptible to the *trans*-10 shift, although the general pattern is also recognizable (Toral et al., 2010; Gómez-Cortés et al., 2011a,b).

Feeds rich in pectic substances and sugars, such as dried citrus pulp (DCP), can be an alternative to cereals favoring a higher acetic/propionic ratio in rumen,

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and a higher rumen pH with a small or null effect on productivity (Bampidis and Robinson, 2006). Reports on the effect of dietary replacement of starch by DCP on biohydrogenation-derived FA in milk are limited to dairy cows (Solomon et al., 2000; Elfert et al., 2006; Cabrita et al., 2007). Studies on dairy ewes involving the dietary replacement of starch sources by DCP or other citrus products are scarce (Fegeros et al., 1995; Castrillo et al., 2004; Jaramillo et al., 2009; Shdaifat et al., 2013) and do not report a detailed milk FA analysis. Furthermore, no reports are available about the effects of replacing cereals by DCP in oil-supplemented diets for dairy ewes.

We hypothesized that replacement of cereal by DCP, in a diet providing a large supply of 18:2n-6, would favor the ruminal *trans*-11 biohydrogenation pathway to the detriment of the *trans*-10 shifted pathway, resulting in higher *cis*-9,*trans*-11–18:2 and *trans*-11–18:1 content in milk. Therefore, we conducted a trial to evaluate the effect of the replacement of cereals by DCP in diets supplemented with soybean oil, on the FA composition of ewe milk, particularly on *trans* octadecenoates and *cis*-9,*trans*-11–18:2.

MATERIALS AND METHODS

Animals, Diets, and Sampling

Four multiparous Serra da Estrela ewes from the flock of the Polo de Investigação of Fonte Boa, with an average parity of 4.8 ± 1.26 ($\pm$ SD) and in the second month of lactation (49 ± 7.6 d after lambing), were used. After weaning at 35 d in milk, ewes were machine milked twice daily at 0900 and 1600 h. The trial occurred between March and April (2014). The ewes were housed in 3.1-m² individual pens, with permanent access to water. The experimental design consisted of a double 2×2 Latin square, with 2 diets and 2 sequential experimental periods of 19 d (14 d of adaptation and 5 d of data and sample collection). The animal handling followed European Union Directive 2010/63/UE concerning animal care.

The diets contained 450 g/kg of corn silage on a DM basis, supplemented with 550 g/kg of either a concentrate meal containing barley and maize or DCP as the main energy source. Both experimental concentrates included 50 g/kg DM of soybean oil to supply plenty of 18:2n-6 as a substrate for rumen biohydrogenation pathways. The concentrate feeds were produced by the Feed Compounds for Animal Unit of Polo de Investigação of Fonte Boa. The ingredients and chemical composition of the diets are presented in Table 1. Dried citrus pulp pellets were prepared using orange juice residues, following a conventional industrial process

Table 1. Composition of the experimental diets

Item	Diet	
	Cereal	Citrus
Ingredient, g/kg of DM		
Maize silage	450	450
Dried citrus pulp	0	240
Barley	190	0
Maize	70	0
Soybean meal, 48%	215	235
Soybean oil	50	50
Sodium bicarbonate	5	5
Calcium carbonate	13	13
Salt	4	4
Minerals and vitamins	3	3
Chemical composition, g/kg of DM		
DM	505	497
Ash	60	71
CP	156	167
Ether extract	76	76
NDF	278	300
ADF	154	184
Sugar	42	190
Starch	377	170
Fatty acid, g/100 g of fatty acids		
16:0	14.7	13.7
18:0	4.0	4.1
<i>cis</i> -9–18:1	23.8	23.6
18:2n-6	51.2	51.6
18:3n-3	6.2	7.0

that includes pressing with addition of calcium oxide, dehydration of extracted liquor, and its incorporation into DCP (Citricos del Andevalo, S.A., Huelva, Spain). The corn silage and the meal fractions of the diets were manually mixed every day, immediately before distribution to the ewes.

Ewes were weighed at the beginning and at the end of the experimental periods. Feed was offered individually ad libitum, considering 10% orts, and intake was controlled daily, weighing the amounts of feed offered and refused. Feed samples were collected on a daily basis for chemical analysis to obtain a composite sample of the diets in each period.

Milk production was registered at every milking. Milk samples were collected daily as 10% of the total volume, obtaining 2 composite samples corresponding to d 15, 16, and 17, and to d 18 and 19 of the experimental periods. Sodium azide solution (0.8%, wt/vol) was used as preservative (0.05 g/L), and milk samples were kept refrigerated before processing for composition, technological properties, and fatty acid analysis.

Chemical Analysis

Feeds were analyzed for DM content (ISO 6496; ISO, 1999), ashes (ISO 5984; ISO, 2002), CP (ISO 5983; ISO, 1997), and ether extract (ISO 6492; ISO, 1999). Neutral detergent fiber, assayed with sodium sulfite,

without α -amylase and expressed with residual ash, and ADF were determined according to Van Soest et al. (1991). Sugar and starch were determined according to Clegg (1956).

The milk was analyzed for fat, protein, lactose, and TS contents (MilkoScan 113B, Foss, Hillerød, Denmark), and pH (Metrohm 713, Herisau, Switzerland). Proportionate samples representing the 2 daily milkings were evaluated for the milk technological properties, assessed by Optigraph (Ysebaert, France), with initial signal set to 7 V, as described by Martins et al. (2009), for the following parameters: clotting time (**R**), rate of firming (**OK20**), and curd firmness measures after 20 min (**A_{20min}**) and 40 min (**A_{40min}**) of the milk coagulation trial, and after the $2 \times R$ (**A_R**) and $3 \times R$ (**A_{2R}**) period, which represent suitable cutting time in soft and hard cheese technologies, respectively.

The milk fat layer was isolated by centrifugation for 15 min at $1,000 \times g$ and stored at -20°C until FA analysis. The milk FAME were prepared by the one-step extraction and transesterification method adapted from Molkentin and Precht (2000). Fatty acids were extracted from 50 mg of milk fat with 1 mL of hexane and 0.2 mL of potassium hydroxide in methanol. After 3 min vortexing and 1 h rest, 100 μL of glacial acetic acid were added, followed by 10 s of vortexing. The liquid phase was removed and transferred to a test tube containing 500 mg of anhydrous sodium sulfate. After 1 min of vortex and 5 min of centrifugation at $1,000 \times g$, the hexane phase was removed and transferred to a vial for gas chromatography. Fatty acid methyl esters were analyzed using a HP6890A chromatograph (Agilent, Avondale, PA), equipped with a flame-ionization detector and fused silica capillary column (SP-2560, 100 m $\times$ 0.25 mm internal diameter $\times$ 0.20 μm film thickness, Supelco, Bellefonte, PA). The injector and detector temperatures were 250 and 280°C , respectively. Initial oven temperature of 100°C was held for 15 min, increased at $10^\circ\text{C}/\text{min}$ to 150°C and held for 15 min, increased at $1^\circ\text{C}/\text{min}$ to 160°C and held for 15 min, and then increased at $1^\circ\text{C}/\text{min}$ to 200°C and held for 45 min. Helium was used as carrier gas at a flow rate of 1 mL/min, the split ratio was 1:30 and 1 μL of sample was injected. Theoretical flame-ionization detector response factors were used to correct peak areas. Milk FA are expressed as a percentage of total FA and as FA yield (g/d) computed from the FA profile and the milk fat content, assuming that FA compose 93.3% of milk fat (Glasser et al., 2007).

Statistical Analysis

Data were analyzed as replicated 2×2 Latin squares using the Mixed procedure of SAS (SAS Institute Inc.,

Cary, NC). The model included the fixed effects of diet and time and the random effects of square, period, and animal within square. The repeated measures over time within the experimental unit (animal within period) were included in the model as the subject for the repeated statement. For the dependent variables with repeated measures over the 5-d experimental period (i.e., intake, milk yield, and FA yields and balances), covariance structures were tested and selected using both Akaike corrected and Bayesian information criteria after adjusting models using the AR(1), ARH(1), ANTE(1), TOEP, TOEPH, CS, CSH, and UN covariance structures. For the dependent variables presenting only 2 repeated measures (i.e., milk composition including FA), UN and CS covariance structures were tested and the best fitting model was selected as described above. Significant differences were considered at the level $P < 0.05$. For graphic representation of the relationship between *trans*-11–18:1 and *cis*-9,*trans*-11–18:2 and *trans*-11–18:1 and *trans*-10–18:1, simple linear regressions were conducted.

RESULTS

Ewes' weight variation, intake, and milk production are presented in Table 2. Average BW was 54 kg for both treatments. Ewes showed a moderate DMI (2.3 and 2.7% live weight for cereal and citrus diets, respectively) and both DMI and FA intake tended ($P < 0.10$) to be higher with the citrus diet. The citrus diet increased ($P < 0.02$) the intake of CP, NDF, and sugar and decreased the intake of starch ($P < 0.001$) when compared with the cereal diet. Milk daily yield was higher ($P = 0.023$) with the citrus diet than with the cereal diet. The daily yield in milk solids increased ($P = 0.034$) with the citrus diet as a result of an increase in lactose yield ($P = 0.012$). Feed efficiency did not differ between diets.

Milk composition (Table 3) was not affected by treatments, but fat tended ($P = 0.074$) to increase when cereals were replaced by DCP. The technological properties of milk for cheese production (Table 3) were not affected by dietary treatments.

The replacement of cereals by DCP (Table 4) decreased ($P < 0.05$) the anteiso-15:0, 18:0, *cis*-9–18:1, and 22:5n-3, proportions in total FA and increased ($P < 0.05$) the 14:0, iso-15:0, *trans*-16:1, *trans*-11–18:1, *trans*-8,*cis*-12–18:2, *cis*-9,*trans*-11–18:2, *trans*-11,*cis*-13–18:2 and *trans*-9,*trans*-11–/*trans*-10,*trans*-12–18:2 proportions in total FA. Replacement of cereals by DCP resulted in a decrease of 3 percentage points for both 18:0 and *cis*-9–18:1, mostly compensated by the 4.12 and 1.84 percentage point increase observed for *trans*-11–18:1 and *cis*-9,*trans*-11–18:2, respectively. The

Table 2. Effect of the diet energy source on nutrient intake, milk yield, and feed efficiency

Item	Diet		SEM	P-value
	Cereal	Citrus		
Live weight, kg	54.0	54.1	8.08	0.714
Live weight variation, %	−0.70	−2.68	0.874	0.205
Intake, g/d				
DM	1,206	1,417	134	0.065
CP	177	247	28.8	0.016
NDF	315	445	51.9	0.014
Starch	459	238	25.9	0.008
Sugars	32	286	33.2	<0.001
Starch + sugar	512	504	49.5	0.832
Fatty acids	83	97	9.3	0.073
Milk yield, mL/d	708	800	127	0.023
Milk solids yield, g/d				
Protein	38.8	42.9	5.33	0.191
Fat	39.2	47.5	8.29	0.143
Lactose	39.7	43.2	6.03	0.012
TS	122	139	15.2	0.034
Feed efficiency				
Milk yield/DMI	0.588	0.601	0.094	0.653
Milk solids yield/DMI	0.106	0.101	0.016	0.807
Milk fat yield/FAI ¹	0.475	0.523	0.108	0.652
Milk protein yield/CPI ²	0.204	0.187	0.030	0.238

¹FAI = fatty acid intake.²CPI = crude protein intake.

ratio *trans*-10–18:1/*trans*-11–18:1 did not differ ($P = 0.53$) between diets, reflecting a very large variability mainly due to the fact that, for the citrus diet, one ewe displayed a very large *trans*-10–18:1/*trans*-11–18:1 ratio compared with the other 3 ewes (4.35 vs. 0.15). The stearoyl-CoA desaturase activity index [*cis*-9–17:1/(*cis*-9–17:1+17:0)] was not affected by treatments ($P = 0.46$).

Intake of C18 FA (Table 5) tended ($P < 0.10$) to be higher with the citrus diet compared with the cereal diet, as a result of the higher intake of 18:0 and 18:3n-3 ($P < 0.05$). Milk output of C18 FA (Table 5)

was higher ($P < 0.030$) with the citrus diet compared with the cereal diet, mostly due to the higher output of *trans*-11–18:1 and *cis*-9,*trans*-11–18:2 as the daily milk output of 18:0, *trans*-10–18:1, *cis*-9–18:1, 18:2n-6 and 18:3n-3 did not differ ($P > 0.10$) between diets. Apparent transfer of dietary C18 FA into milk was not affected by diets.

DISCUSSION

In dairy cows, incorporation of DCP (circa 20% DM) in diets often depresses the DMI (Solomon et al., 2000;

Table 3. Effect of the diet energy source on milk composition and technological traits

Item	Diet		SEM	P-value
	Cereal	Citrus		
Milk solids, %				
Fat	5.15	6.50	1.331	0.074
Protein	5.45	5.21	0.190	0.160
Lactose	5.39	5.32	0.098	0.247
TS	17.0	17.9	1.732	0.531
pH	6.42	6.48	0.046	0.305
Clotting time (R), min	10.1	11.1	1.42	0.393
Curd firmness, ¹ V				
A _R	7.19	6.90	1.190	0.356
A _{2R}	10.8	10.3	1.67	0.411
A _{20min}	6.99	6.18	1.24	0.425
A _{40min}	12.7	12.0	1.58	0.536
Firming rate (OK20), min	9.18	9.62	1.85	0.703

¹A_R and A_{2R} = curd firmness after 2 × R (clotting time) and 3 × R (suitable cutting times for soft and hard cheese technologies, respectively); A_{20min} and A_{40min} = curd firmness after 20 and 40 min, respectively.

Table 4. Effect of the diet energy source on milk fatty acid composition (mg/g of total fatty acids)

Item	Diet		SEM	P-value
	Cereal	Citrus		
4:0	16.5	14.6	6.35	0.142
6:0	17.1	16.3	1.29	0.669
8:0	17.5	17.4	2.39	0.945
10:0	49.8	52.8	8.84	0.554
12:0	29.2	30.4	4.53	0.570
iso-14:0	1.2	0.7	0.31	0.141
14:0	76.8	85.9	4.83	0.040
iso-15:0	1.3	1.7	0.17	0.029
anteiso-15:0	3.6	2.8	0.24	0.018
<i>cis</i> -9-14:1	1.3	1.4	0.31	0.489
15:0	6.2	5.8	0.68	0.060
iso-16:0	2.5	1.9	0.44	0.383
16:0	225	209	7.1	0.069
<i>trans</i> -9-16:1	1.7	5.4	1.51	0.074
iso-17:0	3.4	3.3	0.27	0.369
<i>cis</i> -7-16:1	2.8	2.9	0.25	0.363
<i>cis</i> -9-16:1	7.0	6.4	0.79	0.476
anteiso-17:0	3.7	2.9	0.52	0.254
17:0	3.7	3.6	0.23	0.787
<i>cis</i> -9-17:1	1.3	1.3	0.18	0.757
18:0	134	103	10.1	0.008
<i>trans</i> -6/ <i>trans</i> -7/ <i>trans</i> -8-18:1	6.4	7.7	1.04	0.321
<i>trans</i> -9-18:1	5.6	7.1	0.73	0.202
<i>trans</i> -10-18:1	28.8	22.2	11.43	0.498
<i>trans</i> -11-18:1	24.9	66.1	19.77	0.042
<i>trans</i> -12-18:1	6.7	9.9	1.68	0.077
<i>cis</i> -9-18:1	220	188	18.8	0.011
<i>trans</i> -15-18:1	3.9	4.9	0.33	0.107
<i>cis</i> -11-18:1	8.6	7.9	0.98	0.194
<i>cis</i> -12-18:1	5.2	7.8	1.21	0.138
<i>cis</i> -13-18:1	1.2	1.6	0.19	0.071
<i>trans</i> -16+c14-18:1	4.9	5.7	0.39	0.054
<i>cis</i> -15-18:1	1.2	1.2	0.21	0.959
<i>trans</i> -8, <i>cis</i> -12-18:2	1.6	2.0	0.14	0.046
<i>cis</i> -9, <i>trans</i> -13-18:2	2.4	2.7	0.26	0.339
<i>trans</i> -8, <i>cis</i> -13-18:2	1.4	1.6	0.15	0.309
<i>cis</i> -9, <i>trans</i> -12-18:2	0.7	0.8	0.11	0.320
<i>cis</i> -16-18:1	1.1	1.2	0.11	0.436
<i>trans</i> -9, <i>cis</i> -12-18:2	0.6	0.6	0.15	0.754
<i>trans</i> -11, <i>cis</i> -15-18:2	1.4	1.9	0.27	0.069
18:2n-6	43.2	36.2	11.41	0.437
20:0	2.4	2.2	0.22	0.317
18:3n-3	4.4	4.1	1.14	0.673
<i>cis</i> -9, <i>trans</i> -11-18:2	12.6	31.0	8.22	0.033
<i>trans</i> -9, <i>cis</i> -11-18:2	0.50	0.52	0.062	0.821
<i>trans</i> -10, <i>cis</i> -12-18:2	0.46	0.38	0.268	0.675
<i>trans</i> -11, <i>cis</i> -13-18:2	0.41	0.62	0.073	0.019
<i>trans</i> -11, <i>trans</i> -13-18:2	0.16	0.25	0.039	0.070
<i>trans</i> -9, <i>trans</i> -11-/ <i>trans</i> -10, <i>trans</i> -12-18:2	0.45	0.62	0.051	0.003
20:2n-6	0.25	0.23	0.040	0.103
<i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15-18:3	0.56	0.51	0.118	0.712
22:0	0.93	0.98	0.082	0.701
20:3n-6	0.30	0.28	0.081	0.545
20:3n-3	0.20	0.19	0.040	0.741
20:4n-6	1.8	1.6	0.19	0.269
20:5n-3	0.29	0.27	0.069	0.491
22:5n-3	0.69	0.55	0.079	0.006
22:6n-3	0.38	0.36	0.047	0.793
10-oxo-18:0	3.3	2.2	0.73	0.260
<i>trans</i> -10-18:1/ <i>trans</i> -11-18:1	1.87	0.97	0.950	0.528
SCD ₇ -17 ¹	26.7	25.3	1.20	0.462
SC-SFA ²	211	213	12.7	0.918
SFA	586	558	13.1	0.185
<i>cis</i> -MUFA	250	220	17.9	0.026
<i>trans</i> -MUFA	80	122	13.5	0.005
<i>cis</i> -PUFA	52	44	13.5	0.424
Total ³	117	181	21.4	0.019

¹Stearoyl-CoA activity index computed as $[17:1\text{cis-9}/(17:1\text{cis-9} + 17:0)] \times 100$.²Saturated fatty acid with 14C or less.³Total of biohydrogenation intermediates.

Table 5. Effect of the diet energy source on intake, milk output, and apparent transfer of C18 fatty acids to milk

Item	Diet		SEM	<i>P</i> -value
	Cereal	Citrus		
Intake, g/d				
18:0	3.3	4.0	0.38	0.049
<i>cis</i> -9–18:1	19.8	22.9	2.18	0.084
18:2n-6	42.7	50.2	4.77	0.063
18:3n-3	5.2	6.8	0.64	0.013
Total C18 UFA ¹	67.7	80.3	7.59	0.059
Total C18	71.0	84.0	7.96	0.059
Milk output, g/d				
18:0	5.1	4.3	0.64	0.107
<i>trans</i> -10–18:1	0.95	0.91	0.238	0.885
<i>trans</i> -11–18:1	0.93	3.03	1.121	0.013
<i>cis</i> -9–18:1	8.1	8.1	1.38	0.984
18:2n-6	1.47	1.52	0.222	0.772
<i>cis</i> -9, <i>trans</i> -11–18:2	0.50	1.41	0.487	0.013
18:3n-3	0.15	0.17	0.022	0.107
Total BI ²	4.2	8.1	1.90	0.002
Total C18	19.1	22.7	3.46	0.030
Apparent transfer, %				
18:0	149	118	22.1	0.138
<i>cis</i> -9–18:1	41	38	7.46	0.689
18:2n-6	3.5	3.2	0.41	0.370
18:3n-3	3.0	2.6	0.40	0.236
Total C18	27.4	28.7	4.60	0.715
Milk output/C18 UFA intake, %				
<i>trans</i> -10–18:1	1.44	1.15	0.285	0.353
<i>trans</i> -11–18:1	2.15	3.42	0.807	0.055
<i>cis</i> -9, <i>trans</i> -11–18:2	0.99	1.66	0.375	0.035
Total BI ²	6.6	10.5	2.11	0.034

¹C18 unsaturated fatty acids.²Total of biohydrogenation intermediates.

Santos et al., 2014), probably due to increased rumen fill by hydration of DCP on rumen and slightly lower rumen degradation rate of pectin compared with starch as discussed by Santos et al. (2014). Reports on inclusion of DCP replacing cereals in lactating dairy ewe diets are scarce and do not report a depression in DMI (Fegeros et al., 1995; Castrillo et al., 2004; Shdaifat et al., 2013), suggesting that ewes might respond differently from dairy cows. The DMI results observed for citrus diet are coherent with the observed increase in milk yield and total milk solids. Moreover, the crude efficiency ratios of feed to milk and feed to milk solids remained quite similar between diets. Overall, our results indicate that DCP can replace cereals in dairy ewe diets without negative effects on production, as previously reported by others (Fegeros et al., 1995; Shdaifat et al., 2013).

The inclusion DCP in the ewe diet had no effect on milk solid concentration, except for a tendency for a larger fat percentage. Thus, globally our results confirm that the partial replacement of cereals by DCP had no effect on milk composition of dairy ewes (Fegeros et al., 1995).

Ewe milk is used almost exclusively for cheese production and thus milk technological properties are important quality traits to be evaluated. The replacement of cereals by DCP in the diets did not modify the pH, coagulation, and curd firmness parameters that are within the normal range found in Portuguese breeds (Martins et al., 2009).

The supplementation of ewe diets with PUFA sources has been considered a valuable strategy to obtain cheese enriched in *trans*-11–18:1 and *cis*-9,*trans*-11–18:2 with beneficial effects for human health (Pintus et al., 2013). In dairy cows, basal diets with low fiber and high starch and high PUFA content are associated with a shift in rumen biohydrogenation pathways resulting in strong accumulation of *trans*-10–18:1, to the detriment of *trans*-11–18:1, as a major biohydrogenation intermediate (Aldai et al., 2013). This *trans*-10 shift has been related to the milk fat depression syndrome observed more frequently in dairy cows fed high-concentrate diets (Shingfield and Griinari, 2007). Ewes seem to be less sensitive to changes in forage concentrate ratio of basal diet than cows, when lipid supplements are used (Gómez-Cortés et al., 2011a; Nudda et al., 2014). Even

when fed high concentrate diets, ewes tend to respond to vegetable oil supplementation by largely increasing both *trans*-11–18:1 and *trans*-10–18:1 and presenting a *trans*-10–18:1/*trans*-11–18:1 ratio below 1 (Toral et al., 2010; Gómez-Cortés et al., 2011a,b). In spite of that, in our experiment, milk from ewes fed the cereal diet presented an average value for the *trans*-10–18:1/*trans*-11–18:1 ratio above 1, suggesting the occurrence of an extensive *trans*-10-shift in the rumen. Although not usual, this has been reported in literature (Gómez-Cortés et al., 2008b; Szumacher-Strabel et al., 2011).

Up to 89% of the *cis*-9,*trans*-11–18:2 present in milk from ewes fed vegetable oils is derived from endogenous desaturation of *trans*-11–18:1 catalyzed by stearoyl-CoA desaturase (Toral et al., 2015). As lipid supplementation of ewes fed a high-concentrate diet increases the *trans*-11–18:1 availability, it also linearly increases the *cis*-9,*trans*-11–18:2 in milk (Figure 1). Nevertheless, a much larger *cis*-9,*trans*-11–18:2 concentration in milk should be anticipated if only the *trans*-11 biohydrogenation pathway was expressed in the rumen. Moreover, the high amounts of *trans*-10–18:1 in milk fat found in those situations may compromise its nutritional value (Aldai et al., 2013) and may elicit milk fat depression (Toral et al., 2010).

We had hypothesized that the replacement of cereal starch by DCP in oil-supplemented diets would allow a

higher *cis*-9,*trans*-11–18:2 concentration in milk by preventing the occurrence of *trans*-10–18:1 production in detriment of *trans*-11–18:1. Our results partially support our hypothesis, as *trans*-11–18:1 and *cis*-9,*trans*-11–18:2 concentration in milk increased about 2.5-fold with DCP. However, the increase in *trans*-11–18:1 and *cis*-9,*trans*-11–18:2 was not associated with a clear decrease in *trans*-10–18:1. Similar results have been obtained in dairy cows by Solomon et al. (2000) and Elfert et al. (2006). Solomon et al. (2000) did not report the individualized content of *trans*-10–18:1 and *trans*-11–18:1 in milk but observed an increase of *cis*-9,*trans*-11–18:2 in milk when about 11% of corn was replaced by DCP in a diet that included extruded soybean beans. Elfert et al. (2006) replaced totally corn by DCP in diets with 20% corn and supplemented with 2.25% of soybean oil and observed no effect on *trans*-10–18:1 but a 1.5-fold increase in both *trans*-11–18:1 and *cis*-9,*trans*-11–18:2. Thus, our results are quite similar to those reported by Elfert et al. (2006), which used similar DCP inclusion and the same type of lipid supplement. The large response in milk *trans*-11–18:1 and *cis*-9,*trans*-11–18:2 that we observed is probably explained by the larger soybean oil inclusion (2.25 vs. 5%) and by the differences in the species used in the trials (ewe vs. cow).

The complete replacement of cereals with DCP seems to increase both *trans*-11–18:1 and *cis*-9,*trans*-11–18:2 mostly by promoting an incomplete biohydrogenation pattern, and by tending to favor the biohydrogenation of C18 unsaturated FA through the *trans*-11 pathway, as displayed in Table 5 in which the relation between milk output of C18 FA and intake is shown. High PUFA intake is known to directly increase the amount of biohydrogenation intermediates and thus decrease the completeness of the biohydrogenation but not the extent of biohydrogenation (Harfoot and Hazlewood, 1997). Although the apparent transfer of C18 PUFA is affected by several factors, the major determinant is the extent of its biohydrogenation in the rumen, as discussed in Cabrita et al. (2007). Thus, the C18 unsaturated FA were apparently metabolized to a similar extent in the rumen of animals fed both diets, although in citrus diets a more incomplete biohydrogenation pattern is clear. Replacement of cereals by DCP slightly increased the intake of C18 unsaturated FA. Thus, the effects of replacing cereals by DCP on milk biohydrogenation intermediates output can be partially explained by increased C18 unsaturated FA intake.

Plant secondary compounds have been reported to disturb the rumen biohydrogenation pathways and promote the accumulation of biohydrogenation intermediates, presumably by inhibiting the last reductive step (Vasta and Bessa, 2012). Citrus by-products are rich in plant secondary compounds, such as flavonoids and

Figure 1. Relationship between *trans*-11–18:1 and *cis*-9,*trans*-11–18:2, both expressed in percentage of total milk fatty acids. Data points from cereal (Δ) and citrus (○) treatments. Linear regression: $cis-9,trans-11-18:2 = 0.41 \pm 0.017 \times (trans-11-18:1) + 0.32 \pm 0.097$, $R^2 = 0.98$, $P < 0.001$.

phenolic acids (Tripoli et al., 2007; Santos et al., 2014), and their participation on the modulation of rumen biohydrogenation pathways cannot be excluded, as recently discussed by Lanza et al. (2015). These authors replaced barley by DCP in the diet of growing lambs and also found a reduction of 18:0 and an accumulation in biohydrogenation intermediate concentrations in blood plasma, although DCP inclusion increased simultaneously PUFA and plant secondary compound intake, confounding both factors.

As discussed above, previous reports indicate that in dairy ewes, supplementation of high concentrate diets with PUFA-rich oils generally results in an increase in both *trans*-10-18:1 and *trans*-11-18:1 in milk (Toral et al., 2010; Gómez-Cortés et al., 2011a,b), which suggests that both fatty acids are positively correlated. Nevertheless, we observed a negative relationship between *trans*-11-18:1 and *trans*-10-18:1 (Figure 2) in accordance with the results commonly observed for dairy cows (Shingfield and Grinari, 2007), confirming that a competition between both *trans*-10- and *trans*-11-biohydrogenation pathways also occurs in dairy ewes.

Dried citrus pulp has a low content in starch and a high content in pectin but generally is also quite rich in sugars (Bampidis and Robinson, 2006). In fact, the citrus diet contained much lower starch content than the cereal diet, but this was compensated with an increase in sugar content and the intake of starch plus sugar was quite similar between diets. The occurrence of *trans*-10 shift has been usually related to high-starch, high-oil diets, although much less information is available regarding the effects of sugars. In spite of the very high rumen fermentation rates of sugars, some indications are present that a moderate (5%) replacement of starch by sugars in dairy cow diets may promote intake, mitigate the milk fat depression and increase NDF digestibility, and support the maintenance of fibrolytic microbial population as reviewed by Firkins (2010). The positive effects of moderate replacement of starch by sugars in high-starch diets can mitigate the *trans*-10 shift in dairy cows (Martel et al., 2011). Our results support the concept that starch and sugar might exert a quite differential effect on rumen biohydrogenation, probably by protecting the solid associated fibrolytic and biohydrogenating bacteria. In fact, the citrus diet promoted a higher iso-15:0 and lower anteiso-15:0 in milk than the cereal diets, suggesting that it was able to support a higher fibrolytic and solid adherent community than the cereal diet (Fievez et al., 2012).

The results presented are very promising, but some caution is needed due to the reduced number of animals used in the trial and the large individual variability regarding the expression of the *trans*-10-shift, easily perceived in Figure 2. In fact, 1 in 4 animals

Figure 2. Relationship between *trans*-11-18:1 and *trans*-10-18:1, both expressed in percentage of total milk fatty acids. Data points from cereal (Δ) and citrus (○) treatments. Linear regression: *trans*-10-18:1 = $-1.56 \pm 0.403 \times (\text{trans-11-18:1}) + 8.52 \pm 1.19$, $R^2 = 0.52$, $P < 0.002$.

responded to the citrus diet expressing a large increase of *trans*-10-18:1 in milk, contrasting with the very large increase of *trans*-11-18:1 and *cis*-9,*trans*-11-18:2 observed in the other 3. Moreover, in dairy ewes, the duration of the feeding period on high-concentrate high-oil oil diets has been reported to modify the relative abundance of *trans*-18:1 isomers in milk, mostly by a gradual increase in *trans*-10-18:1 (Gómez-Cortés et al., 2008a, 2011a; Bichi et al., 2013). Our data only considered the average milk FA concentration between d 16 and 19 after the introduction of the diets, so further research is needed to verify if the selective stimulation by the citrus diet of the *trans*-11-18:1 and *cis*-9,*trans*-11-18:2 concentrations in ewe milk will persist throughout time.

CONCLUSIONS

Our data suggest that DCP can be an interesting alternative to cereals in the concentrate fraction of mixed diets for dairy ewes supplemented with vegetable oils. Dried citrus pulp reduces the starch content of the diet, increasing the availability of pectin and sugar as substrates for rumen metabolism, and this seems to favor the *trans*-11 biohydrogenation pathway in the rumen and the content of *trans*-11-18:1 and of *cis*-9,*trans*-11-18:2 in the milk.

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