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Effect of Dietary Fish Oil Supplements on Levels of n-3 Polyunsaturated Fatty Acids, *trans* Acids and Conjugated Linoleic Acid in Ewe Milk

Massimo Mozzon[1], Natale G. Frega[1], Baldassare Fronte[2] and Massimo Tocchini[2]*

[1]Dipartimento di Biotecnologie Agrarie ed Ambientali, Università degli Studi di Ancona, via Brecce Bianche, I-60131 Ancona, Italy

[2]Dipartimento di Produzioni Animali, Università degli studi di Pisa, viale delle Piagge 2, I-56100 Pisa, Italy

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Summary

Three groups of ten lactating Sardinian ewes were used between 3rd and 6th month of lactation to determine the effects of supplementing diets with n-3 fatty acids on milk production and milk fat composition. The control group (A) was fed on a mixture of *Gramineae* hay with a pelleted alfalfa and concentrate; the other groups (B and C) were given the control ration supplemented with graded levels of fish oil. Milk content of nutritionally important fatty acids (n-3 polyunsaturated fatty acids, PUFA; *trans* acids and conjugated linoleic acid, CLA) was determined by gas chromatography. Unlike cow milk, milk of normally fed ewes contained detectable quantities of n-3 PUFA and higher amounts of CLA. The supplementation with fish oil resulted in an increased milk fat levels of n-3 PUFA, CLA and *trans* isomers. There was a positive correlation (R^2 = 0.964) between the *trans* and n-3 fatty acid content in ewe milk, and a close positive correlation (R^2 = 0.996) between CLA and *trans* monoenoic fatty acid contents. PUFA supplementation resulted in a slightly, but not statistically significative, decrease of milk fat percentage. On the contrary, dietary fish oil markedly affected milk production: ewes fed on the n-3 supplemented diet produced more milk than the ewes fed with the control diet.

Key words: ewe milk fat, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), fish oil, conjugated linoleic acid (CLA), *trans* fatty acids

Introduction

Contemporary nutritional literature abounds with arguments about the role of n-3 polyunsaturated fatty acids (PUFA) as a factor influencing our health, in particular their role in cardiovascular diseases (*1–5*), in neurological diseases (retinitis pigmentosa, depression, schizophrenia, Alzheimer's disease) (*6–10*), in inflammatory diseases (rheumatoid arthritis, Crohn's disease, ulcerative colitis) (*11–15*), in the developing of nervous system (*16–19*), in fertility (*20*), and towards some tumors (*21–23*). The widely accepted opinion is undoubtedly that the health of people would be improved both by a reduction in fat intake and by a change in dietary intake of fatty acids, avoiding the saturates and prefering the polyunsaturates. Also, the recognition of specific metabolic roles of the longer chain C20 and C22 acids has drawn attention to the importance of a more

* Corresponding author; Phone: ++39 (0)71 22 04 926; Fax ++39 (0)71 22 04 980; E-mail: mm@popcsi.unian.it

balanced nutritional strategy between the provision of n-3 and n-6 acids (*24*).

A good way to raise the n-3 PUFA content in the diet, without radical changes of eating habits, seems to be the enrichment of frequent and commonly consumed food products. New terms such as medical, designer, and functional foods have infiltrated the marketing and scientific literature for designation of these products. The manipulation of dietary lipid composition can be carried out in two ways: directly, through the incorporation of n-3 PUFA sources (marine oils, marine unicellular algae) in foodstuffs; and indirectly, through the employment of the same sources of n-3 fatty acids in feed integration of farm animals (ruminants, swines, poultry), in which case PUFA will become biologically transferred in the lipid fraction of animal tissues (meats) and of edible animal products (milk, eggs) (*25–33*).

Ewe milk and dairy products have interesting peculiarity in their organoleptic and nutritional characteristics (*34,35*). This explains the growing world wide market demand for ewe (and goat) milk products by gourmet restaurants and food stores and by people afflicted with allergies to cow milk. Through the nutritional betterment of their lipid fraction composition further exploitation of these products could thus represent a good commercial opportunity.

The purpose of this study was to evaluate the content of nutritionally important fatty acids (n-3 PUFA, *trans* acids and conjugated linoleic acid) in ewe milk at varying dietary fish oil levels.

Material and Methods

Materials

Pure analytical standards of fatty acid methyl esters were purchased from Sigma Chemical Co. (St. Louis, MO) and Supelchem Inc. (Bellefonte, PA). Analytical grade solvents were purchased from BDH Ltd. (Poole, UK).

Ewe feeding management and milk sampling

A 3-month trial was performed with 3 groups of multiparous Sardinian ewes (10 subjects each) in the 3rd month of lactation, kept in adjoining box on permanent litter. The animals were fed *ad libitum* amounts of hay (80 % *Gramineae*) and received a daily supplement of 0.5 kg pelleted alfalfa and 1.0 kg concentrate (provided by Trouw Nutrition Italia S.p.A., Bussolengo, Italy). The differences between the rations of the 3 groups regarded concentrate composition: groups B and C were fed with concentrate to which 3 and 4.5 % of premixed n-3 integrator were added, respectively; while the control group (A) did not receive n-3 supplementation. Milking was carried out separately for each group. Milk samples were taken at the beginning (0 d) and at days 20, 40, 60, and 80 of the experiment. They were immediatly frozen and stored at –20 °C. Ewe milk yield was recorded daily.

Fatty acid analysis

Total lipids were extracted from individual milk samples according to the method of Roese-Gottlieb (*36*). A proper quantity of hexane solution of methyl nonadecanoate was previously added to the samples as internal standard. Fatty acid methyl esters were prepared by alkaline transesterification (*37*): free fatty acids were previously esterified with an ethereal solution of diazomethane, prepared according to Fieser and Fieser (*38*). Fatty acid methyl esters were analysed by capillary gas chromatography (HRGC) with a Chrompack (Middelburg, The Netherlands) CP-9002 instrument equipped with a splitter/splitless injection system and a flame ionization detector. The operative parametres are in Table 1. Peaks identification was carried out by comparing retention times (RTs) with those of known reference material and by comparison with the results published in literature (*39,40*). For the identification of branched chain fatty acids, we had recourse to the aid of catalytic hydrogenation. The reaction was performed by bubbling pure hydrogen in a test-tube containing a small amount of the hexane solution of fatty acid methyl esters prepared for the HRGC analysis and a few milligrams of platinum oxide powder (according to Adams) as catalyst (*41*). The presence of spurious peaks (unsaponifiable components, transesterification by-products) was verified through fatty acid methyl esters purification by thin layer chromatography (TLC) on 200 × 200 × 0.5 mm silica gel G plates (Carlo Erba, Milano, Italy); *n*-hexane:diethyl ether 60/40 was the developing solvent, and 0.2 % ethanolic solution of 2,7′-dichlorofluorescein sodium salt was the detection reagent. Fatty acid composition of concentrate feed lipid fractions were analysed by HRGC under the same experimental conditions summarised above. Fatty acids were obtained by direct saponification of feed samples (*42*), to which a proper quantity of hexane solution of methyl nonadecanoate was previously added as internal standard. The fatty acids were methylated by treatment with an ethereal solution of diazomethane (*38*).

Table 1. Experimental conditions used in gas chromatographic determinations

Parametre	Description
Stationary phase	CP Sil 88a
Column internal diameter/mm	0.25
Column lenght/m	100
Column film thickness/μm	0.2
Carrier gas	He
Column pressure/KPa	250
Oven temperature	50 °C (1 min) 10 °C/min→ 170 °C
	170 °C (27 min) 5 °C/min → 230 °C (20 min)
Detector temperature/°C	250
Injector temperature/°C	250
Injector purge delay time/min	0.5
Split ratio	150:1
Electronic pressure control mode	Constant flow

[a] Chrompack (Middelburg, The Netherlands)

Statistical methods

Statistical treatment of experimental data was done by two-way analysis of variance (ANOVA). All the analyses were performed using the statistical software Prism (GraphPad Software Inc., San Diego, CA).

Results and Discussion

Gas chromatographic analyses of feed lipid fractions confirmed the absence of n-3 long chain polyenoic fatty acids (LCP) (eicosapentaenoic acid, EPA; docosapentaenoic acid, DPA; docosahexaenoic acid, DHA) in the control feed (group A) (Table 2). Experimental data showed that the n-3 source was a fractionated fish oil: saturated fatty acids did not exceed 8 % of total fatty acids. Premixed feeds employed in our experiments were obtained by mixing fish oil with melted palm stearin and silica; the mixture was then cooled by means of a spray-drying technology. Final product resulted in microspheres in which the superficial exposition of the polyunsaturated fatty acids had been considerably reduced. Concentrate feed to which 3 % of premixed n-3 integrator was added (group B) contained 0.71, 0.25, and 1.12 g/kg of EPA, DPA, and DHA, respectively. Concentrate feed to which 4.5 % of premixed n-3 integrator was added (group C) contained 1.43, 0.49 and 2.21 g/kg of EPA, DPA, and DHA, respectively.

Table 2. Fatty acid profiles of fish oil (n-3 source) and premixed integrators

Fatty acid	FA (% of total FA) in			
	fish oil	premix A	premix B	premix C
14:0	1.0	0.1	0.4	0.6
16:0	3.1	15.0	16.1	17.3
16:1 Δ^9c	1.4	0.1	0.2	0.3
17:0	n.d.	0.1	0.2	0.2
18:0	1.4	2.0	13.2	20.5
18:1 Δ^9c + Δ^{11}c	3.9	25.4	18.0	15.3
18:2 $\Delta^{9,12a}$	0.5	53.0	41.9	32.9
20:0	1.2	0.4	0.6	0.8
20:1 Δ^{11}c	1.4	0.4	0.4	0.3
α-18:3[a]	0.8	2.4	3.1	3.1
20:2 $\Delta^{11,14a}$	0.4	0.1	0.1	0.1
22:0	0.6	0.3	0.3	0.3
22:1 Δ^{13}c	0.8	n.d.	n.d.	n.d.
20:3 $\Delta^{11,14,17a}$	0.6	n.d.	n.d.	n.d.
20:4 $\Delta^{5,8,11,14}$ (AA)[a]	1.5	n.d.	0.3	0.5
20:5 $\Delta^{5,8,11,14,17}$ (EPA)[a]	21.6	n.d.	1.5	2.4
24:1 Δ^{15}c	1.9	n.d.	n.d.	n.d.
22:5 $\Delta^{7,10,13,16,19}$ (DPA)[a]	8.7	n.d.	0.6	0.8
22:6 $\Delta^{7,10,13,16,19}$ (DHA)[a]	40.3	n.d.	2.3	3.7
O.P.	8.9	0.7	0.8	0.9

n:m = number of carbon atoms:number of double bonds
Δ = position of the double bonds
c/t = *cis*/*trans* configuration of double bonds
[a]All *cis* isomers
nd = not detectable
O.P. = other peaks

The fatty acid contents of the ewe milk samples, in reference to milk fat weight are presented in Table 3. Short-chain fatty acid (from 4 to 12 carbon atoms) data were barely reproducible because of the injection system employed, and were not presented. Unlike cow milk, fatty acid data of group A showed that milk of normally fed ewes contained detectable quantities of n-3 LCP and higher amounts of conjugated linoleic acid isomers (CLA): DPA content ranged between 0.5 and 0.7 mg/g milk fat (corresponding to 3.2–4.6 mg/100 g of whole milk); EPA content varied from 0.3 to 0.4 mg/g milk fat (corresponding to 2.1–2.6 mg/100 g of whole milk); CLA content lay in the range betwen 6.6–12.9 mg/g of milk fat and 37.1–92.1 mg/100 g of whole milk. At 0 d there were no significant differences in milk fat composition among the three dietary treatment groups. Over the experimental period, major alterations in milk fat composition were detected between the first (0 d) and the second (20 d) sampling. Oleic, palmitic, and stearic acids were the most abundant fatty acids and they showed similar trends in all ewe groups. Saturated fatty acids, palmitic and stearic, were present at the same level in the first milk samples and exhibited a diverging trend in the following samplings: palmitic acid increased in the same manner in all ewe groups, while stearic acid decreased between 0 d and 40 d to an extent that was in the order C > B > A, and then slightly increased. A similar trend was observed for oleic acid. It must be pointed out that, in our operative conditions, oleic acid coeluted with *trans*-15 18:1. However, this isomer increases to an average of only 5 % of total *trans* 18:1 acids (*39*), thus we disregarded the contribution of 18:1 Δ^{15} to peak area of oleic acid.

The n-3 LCP levels in milk fat of group A ewes did not show significant differences throughout the experimental period. The supplementation with fish oil resulted in an increased milk fat concentrations of n-3 LCP in the first three weeks of dietary treatment, afterwards LCP levels did not significantly change. Both, time and dietary treatment, significantly affected EPA, DPA, and DHA concentrations in milk fat ($P<0.0001$). The highest levels of n-3 LCP were reached in milk of group C (7.1–8.1 mg/g milk fat), which were about twice the amount of those found in milk of group B (4.0–4.9 mg/g milk fat). This finding was in agreement with n-3 fatty acid contents of the supplemented diets, as reported above. α-linolenic acid (LnA) is the biological precursor of the n-3 fatty acid family. A slight increase of LnA levels in milk fat of ewes fed with both the normal and supplemented diets was observed. Compared to group A, milk fat of group C ewes had a slightly higher LnA levels (3.5–4.6 mg/g of milk fat *vs.* 3.6–4.3, $P<0.005$), while milk fat of group B ewes had the highest concentration of LnA (4.2–5.1 mg/g of milk fat, $P<0.0001$). Statistical analysis between groups A and B showed that there was a parallel trend in LnA levels (time/treatment interaction not significant). Unlike the results obtained previously in laying hens (*43*,*44*), fish oil supplementation to lactating ewes did not result in a decrease of arachidonic acid (AA) in milk fat. Ewes fed with the normal diet doubled AA levels from 0.6 to 1.2 mg/g milk fat, while AA levels were slightly, but not

Table 3. Fatty acid (FA) levels in milk fat (mean ± SD of three replicates) of the three ewe groups (A-C)

Fatty acid	FA content (mg/g milk fat) after														
	0 d			20 d			40 d			60 d			80 d		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
14:0	16.5 ± 3.8	19.0 ± 3.5	21.1 ± 3.8	22.4 ± 4.5	20.1 ± 3.8	25.8 ± 4.4	26.2 ± 4.3	26.9 ± 4.5	27.5 ± 4.8	23.6 ± 3.5	23.9 ± 4.1	24.3 ± 3.9	24.1 ± 3.8	28.3 ± 5.3	26.6 ± 4.2
iso 15:0	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
*a-iso*15:0	1.5 ± 0.3	1.9 ± 0.3	2.0 ± 0.3	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.1	1.2 ± 0.2	1.3 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	1.1 ± 0.1
14:1 Δ^9c	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
15:0	2.9 ± 0.2	3.5 ± 0.4	3.5 ± 0.4	2.5 ± 0.3	3.0 ± 0.2	2.7 ± 0.2	2.5 ± 0.3	3.4 ± 0.2	2.9 ± 0.2	2.7 ± 0.3	3.4 ± 0.2	2.7 ± 0.3	2.8 ± 0.2	3.5 ± 0.3	3.1 ± 0.2
iso 16:0	0.8 ± 0.2	1.2 ± 0.2	0.9 ± 0.1	0.8 ± 0.2	0.9 ± 0.1	0.8 ± 0.2	0.7 ± 0.2	1.0 ± 0.1	0.8 ± 0.2	0.8 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.2	0.9 ± 0.1	0.9 ± 0.2
16:0	73.7 ± 3.8	86.6 ± 4.5	80.8 ± 4.1	79.2 ± 3.9	92.1 ± 5.0	91.9 ± 4.9	89.1 ± 4.8	111.9 ± 6.9	99.1 ± 5.8	96.5 ± 5.3	107.6 ± 6.5	102.9 ± 5.3	90.7 ± 3.8	109.2 ± 6.7	108.6 ± 6.5
16:1 Δ^9t + *iso* 17:0	2.0 ± 0.1	2.3 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	2.5 ± 0.2	2.3 ± 0.1	1.8 ± 0.1	2.3 ± 0.2	2.0 ± 0.1	1.8 ± 0.1	2.2 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	2.4 ± 0.1	2.4 ± 0.1
16:1 *trans*[a]	2.2 ± 0.1	2.9 ± 0.2	2.2 ± 0.1	1.5 ± 0.2	3.4 ± 0.3	2.8 ± 0.2	1.4 ± 0.1	2.8 ± 0.2	3.1 ± 0.2	1.3 ± 0.1	2.7 ± 0.3	3.7 ± 0.2	1.6 ± 0.1	2.6 ± 0.3	3.1 ± 0.2
anteiso-17:0	2.8 ± 0.3	3.2 ± 0.3	2.8 ± 0.3	1.9 ± 0.1	2.3 ± 0.2	1.8 ± 0.1	2.0 ± 0.1	2.3 ± 0.2	1.8 ± 0.1	1.9 ± 0.1	2.2 ± 0.1	1.8 ± 0.2	2.0 ± 0.1	2.4 ± 0.1	2.1 ± 0.1
16:1 Δ^9c	2.5 ± 0.5	2.9 ± 0.2	2.6 ± 0.7	2.6 ± 0.7	2.6 ± 0.8	2.1 ± 0.4	3.4 ± 1.1	3.6 ± 1.4	2.9 ± 0.4	3.7 ± 1.0	3.8 ± 1.5	3.2 ± 0.8	3.5 ± 0.5	3.1 ± 1.8	3.3 ± 0.5
17:0	3.1 ± 0.3	3.5 ± 0.3	3.1 ± 0.3	2.7 ± 0.2	3.7 ± 0.3	3.0 ± 0.2	2.6 ± 0.2	3.5 ± 0.3	2.9 ± 0.2	2.5 ± 0.2	3.7 ± 0.2	3.0 ± 0.2	2.5 ± 0.1	3.8 ± 0.1	3.7 ± 0.2
17:1 Δ^9c	0.6 ± 0.2	1.1 ± 0.1	0.7 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0.8 ± 0.1
18:0	73.2 ± 3.5	85.4 ± 4.5	79.5 ± 3.8	56.3 ± 2.5	40.6 ± 1.8	30.4 ± 1.2	49.8 ± 2.0	44.7 ± 1.5	23.4 ± 1.0	50.6 ± 2.1	44.1 ± 1.6	28.9 ± 5.3	53.4 ± 2.3	48.6 ± 1.9	38.5 ± 2.3
18:1 Δ^6t + Δ^7t	1.4 ± 0.2	1.6 ± 0.2	2.3 ± 0.2	2.0 ± 0.1	3.5 ± 0.2	2.7 ± 0.2	1.9 ± 0.1	3.2 ± 0.2	2.1 ± 0.1	1.8 ± 0.1	3.7 ± 0.1	2.5 ± 0.2	1.9 ± 0.1	3.4 ± 0.1	3.4 ± 0.1
18:1 Δ^9t	1.7 ± 0.3	1.8 ± 0.2	2.6 ± 0.2	2.1 ± 0.2	4.9 ± 0.2	4.9 ± 0.2	2.0 ± 0.1	4.1 ± 0.1	4.3 ± 0.2	1.9 ± 0.1	4.2 ± 0.1	4.5 ± 0.2	2.0 ± 0.1	4.1 ± 0.1	5.3 ± 0.1
18:1 Δ^{11}t + Δ^{10}t	24.9 ± 2.3	30.9 ± 3.1	23.6 ± 2.1	13.9 ± 0.6	41.4 ± 1.4	60.9 ± 4.1	11.8 ± 0.5	31.5 ± 1.3	53.5 ± 2.2	12.2 ± 0.5	29.5 ± 1.1	52.6 ± 2.3	12.6 ± 0.6	27.9 ± 1.0	51.3 ± 2.1
t + c 18:1[b]	5.1 ± 0.2	6.5 ± 0.2	8.2 ± 0.3	6.0 ± 0.2	11.9 ± 0.3	10.9 ± 0.5	5.5 ± 0.1	10.2 ± 0.3	8.8 ± 0.3	5.4 ± 0.1	11.5 ± 0.3	9.1 ± 0.3	5.5 ± 0.1	11.1 ± 0.3	12.2 ± 0.3
18:1 Δ^9c[c]	120.7 ± 5.5	128.1 ± 5.9	122.8 ± 5.8	105.3 ± 4.4	83.8 ± 3.8	62.8 ± 4.3	108.7 ± 4.3	94.3 ± 4.3	57.5 ± 3.8	110.6 ± 4.5	96.0 ± 4.2	70.1 ± 2.8	112.1 ± 5.7	109.4 ± 4.3	91.2 ± 3.5
18:1 Δ^{11}c	2.0 ± 0.2	2.5 ± 0.2	2.1 ± 0.2	2.0 ± 0.2	3.0 ± 0.3	3.8 ± 0.2	2.1 ± 0.2	2.8 ± 0.3	3.2 ± 0.2	2.2 ± 0.2	2.9 ± 0.3	3.4 ± 0.2	2.1 ± 0.2	3.0 ± 0.3	3.6 ± 0.2
18:1 Δ^{12}c	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	2.2 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	2.5 ± 0.2	1.4 ± 0.1	0.5 ± 0.1	2.9 ± 0.2	1.7 ± 0.1	0.8 ± 0.1	2.7 ± 0.2	1.9 ± 0.1	1.3 ± 0.1
18:1 Δ^{13}c	0.2 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	0.7 ± 0.1
isom. 18:2[d,f]	8.3 ± 1.5	9.8 ± 1.4	8.1 ± 1.5	4.9 ± 0.3	8.6 ± 1.5	6.3 ± 0.9	5.5 ± 0.3	8.7 ± 1.5	7.7 ± 1.1	6.0 ± 0.4	9.1 ± 1.7	8.4 ± 1.5	6.2 ± 0.4	9.2 ± 1.7	10.5 ± 1.5
18:2 $\Delta^{9,12}$[f]	7.4 ± 0.8	8.1 ± 1.3	7.9 ± 1.3	15.7 ± 0.8	14.1 ± 0.5	12.3 ± 0.5	17.5 ± 0.8	14.6 ± 0.5	13.2 ± 0.5	18.7 ± 0.9	15.8 ± 0.6	14.7 ± 0.5	19.3 ± 0.8	17.0 ± 0.8	15.7 ± 0.6
20:0	2.0 ± 0.3	2.8 ± 0.3	2.3 ± 0.2	3.7 ± 0.1	4.7 ± 0.6	5.2 ± 0.3	3.9 ± 0.1	5.8 ± 0.5	5.5 ± 0.3	3.9 ± 0.1	5.3 ± 0.3	5.0 ± 0.3	4.1 ± 0.1	6.6 ± 0.4	6.1 ± 0.4
20:1 Δ11c	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	1.5 ± 0.2	1.8 ± 0.3	0.5 ± 0.1	1.5 ± 0.2	1.4 ± 0.3	0.6 ± 0.1	0.9 ± 0.2	1.7 ± 0.3	0.6 ± 0.1	1.3 ± 0.3	2.0 ± 0.4
α-18:3[f]	3.7 ± 0.2	4.2 ± 0.2	3.5 ± 0.2	3.6 ± 0.2	4.1 ± 0.2	3.7 ± 0.3	3.9 ± 0.2	4.7 ± 0.2	4.5 ± 0.3	4.0 ± 0.2	4.9 ± 0.2	4.6 ± 0.3	4.3 ± 0.1	5.1 ± 0.2	4.6 ± 0.3
CLA[e]	12.9 ± 0.8	14.7 ± 0.9	14.0 ± 0.9	6.6 ± 0.5	19.3 ± 0.9	27.1 ± 1.1	6.8 ± 0.5	15.9 ± 1.0	28.1 ± 1.1	6.8 ± 0.5	15.9 ± 0.9	29.6 ± 1.2	6.9 ± 0.5	15.8 ± 0.9	29.8 ± 1.1
22:0	0.9 ± 0.1	1.1 ± 0.1	1.5 ± 0.3	1.2 ± 0.3	1.7 ± 0.3	2.3 ± 0.3	1.4 ± 0.4	1.6 ± 0.3	2.1 ± 0.4	1.5 ± 0.4	1.6 ± 0.3	2.5 ± 0.5	1.3 ± 0.4	2.1 ± 0.5	2.4 ± 0.4
20:3 $\Delta^{11,14,17}$[f]	nd	nd	nd	nd	nd	0.2 ± 0.0	nd	nd	0.2 ± 0.0	nd	0.3 ± 0.1	0.3 ± 0.2	nd	0.2 ± 0.2	0.6 ± 0.1
20:4 $\Delta^{5,8,11,14}$ (AA)[f]	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	0.9 ± 0.0	0.8 ± 0.1
22:2 $\Delta^{11,14}$[f]	0.2 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	0.5 ± 0.1	0.7 ± 0.1	0.1 ± 0.0	0.5 ± 0.2	0.5 ± 0.0	0.2 ± 0.0	0.3 ± 0.2	0.6 ± 0.1
24:0	nd	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.2	0.7 ± 0.2
20:5 $\Delta^{5,8,11,14,17}$ (EPA)[f]	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.7 ± 0.1	1.6 ± 0.3	0.3 ± 0.1	1.0 ± 0.1	1.6 ± 0.2	0.4 ± 0.0	1.0 ± 0.1	1.8 ± 0.2	0.4 ± 0.1	1.1 ± 0.0	1.7 ± 0.1
24:1 Δ^{15}c	nd	nd	nd	nd	1.1 ± 0.2	1.0 ± 0.1	nd	0.3 ± 0.1	0.6 ± 0.1	nd	0.3 ± 0.0	0.7 ± 0.1	nd	0.5 ± 0.2	0.6 ± 0.1
22:5 $\Delta^{7,10,13,16,19}$ (DPA)[f]	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.2	0.7 ± 0.1	1.4 ± 0.1	2.5 ± 0.2	0.5 ± 0.1	1.2 ± 0.1	2.3 ± 0.2	0.6 ± 0.1	1.1 ± 0.1	2.4 ± 0.2	0.7 ± 0.1	1.3 ± 0.2	2.2 ± 0.2
22:6 $\Delta^{4,7,10,13,16,19}$ (DHA)[f]	tr	tr	tr	tr	1.7 ± 0.2	3.6 ± 0.2	tr	1.9 ± 0.3	3.5 ± 0.3	tr	1.9 ± 0.5	3.8 ± 0.3	tr	2.4 ± 0.4	3.3 ± 0.3

n:m = number of carbon atoms:number of double bonds; Δ = position of the double bonds; c/t = *cis*/*trans* configuration of double bonds

[a]Group of several peaks corresponding to *trans* isomers of 16:1; [b]Sum of all peaks corresponding to a mixture of *cis* and *trans* 18:1 acids (Δ^6c + Δ^7c + Δ^{12}t + Δ^{13}t + Δ^{14}t)
[c]Isomer 18:1 Δ^{15}t overlap with oleic acid; [d]Group of several peaks corresponding to *cis* and/or *trans* isomers of octadecadienoic acids
[e]Conjugated 18:2 acids; [f]All *cis* isomers, tr = traces; nd = not detectable

significantly, increased for ewes fed with the n-3 supplemented diet.

Natural *trans*-18:1 and 16:1 isomers are present in all ruminant milk and meat fats as a result of biohydrogenation by rumen bacteria of dietary polyunsaturated fatty acids. Interest in *trans* fatty acids has grown owing to concerns that high intakes may increase the relative risk of developing heart disease (*45*,*46*). All *trans*-16:1 isomers with their ethylenic bond between positions Δ^4 and Δ^{14}, and all *trans*-18:1 isomers with their ethylenic bond between positions Δ^6 and Δ^{16} were observed in ewe milk (*39*,*40*). However, *trans*-16:1 isomers only represented 7.4–8.8 %, 6.6–7.7 %, and 3.9–7.1 % of the sum *trans*-16:1 plus *trans*-18:1 acids in milk fat of ewe groups A, B, and C, respectively. Moreover, although the *trans*-10 and *trans*-11 isomers were incompletely separated, HRGC traces showed that the main component was *trans*-11 18:1 (vaccenic acid). The content of total *trans* fatty acids of milk samples varied between the first (0 d) and the third (40 d) sampling: it decreased from 30.1 to 17.1 mg/g milk fat for ewes fed with control diet (group A); it increased from 37.2 to 53.2 and then decreased to 41.5 mg/g of milk fat for ewes fed with supplemented diet B; it increased from 30.7 to 71.2 and then decreased to 62.9 mg/g of milk fat for ewes fed with supplemented diet C. After 40 days of dietary treatments, the content of total *trans* fatty acids of milk samples was kept nearly constant at levels of 17.1–18.1, 38.0–41.5 and 62.9–63.3 mg/g of fat in ewe milk samples of groups A, B and C, respectively. Our experimental data showed a positive correlation ($R^2 = 0.964$) between the *trans* and n-3 LCP fatty acid content in ewe milk. Therefore, the dietary supplementation of lactating ewes with a PUFA source resulted both in an increase of nutritionally valuable fatty acids (n-3 LCP) and an increase of negative fatty acids (*trans* isomers) in milk fat. Procedures involving protection of PUFA against rumen hydrolysis and subsequente biohydrogenation have been extremely successful in the promotion of tissue and product fatty acid quality. Several encapsulation technologies and wall materials (saccharides, proteins, surfactants) have been developed to this end (*47–49*). Premixed n-3 integrator utilized in our trial was microencapsulated to limit the extent of rumen hydrogenation processes, however PUFA protection was not complete, as demonstrated by increased *trans* fatty acid content.

It seems ironic that a feature of rumen metabolism could be both negative (production of *trans* isomers) and positive (production of CLA). In fact, conjugated linoleic acid isomers are prominent intermediary of rumen biohydrogenation, and it has been reported that they are associated with the reduction of chemically induced cancers in animals, they are antiatherogenic and have an effect on body fat and energy metabolism (*50*,*51*). The major isomer found in ewe milk was *cis*-9,*trans*-11 linoleic acid, amounting to as much as 89–93 % of the total CLA content. The absence of coelution between CLA and Δ^5, Δ^8 and Δ^{11} 20:1 isomers was verified under the adopted gas chromatographic conditions. At 0 d there were no significant differences in fat milk CLA contents among the three dietary treatment ewe groups (12.9–14.7 mg/g of milk fat). For ewes fed with the supplemented diet B, CLA in milk fat remained constant at the initial level; between 0 d and 20 d, CLA levels halved in the control group and doubled in the group C, when they remained constant. As expected, there was a close positive correlation ($R^2 = 0.996$) between CLA and *trans* monoenoic fatty acid contents.

Fatty acid contents of whole milk were affected by ewe milk fat percentage (Table 4). Milk fat content showed similar trend for each group: it decreased in the first 40 days of trial, then slightly increased. Fish oil supplementation resulted in a slight, but not statistically significant, decrease of milk fat percentage. Instead, dietary treatments markedly affected milk production (Table 5): ewes fed with the n-3 supplemented diet produced more milk than the ewes fed with the control diet. Table 6 summarises the nutritionally important fatty acid contents in the milk of the three ewe groups. As it can be observed, fish oil supplementation strongly increased *trans* fatty acids levels in milk. This result points out that rumen biohydrogenation should not be disregarded: as previously demonstrated (*39*), the contribution of ewe (and goat) milk fat to the daily intake of *trans* fatty acids may not be negligible in the diet of European people.

Table 4. Fat content of milk samples

	ω (milk fat)/ % after				
	0 d	20 d	40 d	60 d	80 d
Group A	7.1	6.5	5.5	6.1	5.9
Group B	6.9	6.0	5.2	5.9	5.6
Group C	6.8	5.4	5.0	5.2	5.3

Table 5. Milk yield in the three ewe groups during experimental period

	Milk production / L				
	0–20 d	20–40 d	40–80 d	Total	Var. %
Group A	150	200	360	710	–
Group B	150	200	400	750	+6
Group C	150	210	480	840	+18

Conclusions

Recognition of the role of food for improving and preserving health is having an increasing diffusion among consumers. New terms such as medical, designer and functional foods have infiltrated the marketing and scientific literature for designation of products which contain health-promoting components, both naturally present or added. Fat manipulated milk could be regarded as functional food. A vast array of experiments have been undertaken to enhance milk PUFA levels, however it has been only through protection of the PUFA sources that true substantial increases could be obtained. Besides, the efficiency of the protection system affects the milk content of *trans* isomers of fatty acids, which could be potentially positive (CLA) or potentially

Table 6. Nutritionally important fatty acid (FA) contents (mean ± SD of three replicates) in the whole milk of the three ewe groups (A-C)

Fatty acid	FA content (mg/100 g whole milk) after														
	0 d			20 d			40 d			60 d			80 d		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
CLA[a]	92.1 ± 4.1	98.1 ± 5.0	99.0 ± 4.4	43.0 ± 2.8	115.5 ± 5.5	151.7 ± 6.1	37.1 ± 2.5	85.6 ± 4.8	146.7 ± 5.8	39.1 ± 2.2	97.8 ± 4.9	160.4 ± 6.3	38.2 ± 2.4	91.8 ± 5.6	156.7 ± 6.1
EPA[b]	2.6 ± 0.6	2.9 ± 0.5	2.2 ± 0.3	2.6 ± 0.5	4.2 ± 0.8	9.2 ± 1.2	2.1 ± 0.6	5.4 ± 0.7	8.6 ± 1.4	2.3 ± 0.6	6.2 ± 0.5	10.0 ± 0.6	2.1 ± 0.8	6.2 ± 0.3	9.2 ± 0.6
DPA[c]	3.6 ± 0.8	4.3 ± 0.9	4.8 ± 0.5	4.6 ± 0.8	8.1 ± 1.0	13.8 ± 1.1	3.2 ± 0.8	6.3 ± 0.7	11.9 ± 0.7	3.2 ± 0.9	6.5 ± 0.6	12.9 ± 0.8	3.5 ± 0.9	7.8 ± 1.2	11.4 ± 0.6
DHA[d]	tr	tr	tr	tr	10.2 ± 1.3	20.0 ± 0.8	tr	10.1 ± 1.5	18.3 ± 2.5	tr	11.5 ± 1.9	20.3 ± 1.0	tr	13.8 ± 2.2	17.5 ± 1.5
trans[e]	198.6 ± 6.4	228.7 ± 7.3	201.7 ± 6.5	117.7 ± 5.6	298.8 ± 11.5	383.3 ± 15.1	98.3 ± 4.9	208.1 ± 7.9	312.0 ± 12.4	90.5 ± 4.8	229.9 ± 8.3	322.6 ± 13.3	95.4 ± 6.1	205.9 ± 6.7	315.8 ± 12.7

[a] Conjugated linoleic acid isomers
[b] Eicosapemtaenoic acid
[c] Docosapemtaenoic acid
[d] Docosahexaenoic acid
[e] Sum *trans*-16:1 plus *trans*-18:1 acids

dangerous (C16 and C18 monoenoic). Receptiveness to changes in fat quality differs extensively among species, breeds and, to some extent, among subjects. Changes in fat composition may also affect other metabolic features or need to be accompanied by associated compositional alteration in order to maximise nutritional benefits: *eg*. increased milk polyunsaturation may require appropriate increases in antioxidant capacities. However, with all animal species there has to be an acceptance of the limits to change based on a combination of the animal itself, consumer requirements and straightforward economics.

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Utjecaj dijetalnog ribljeg ulja na razinu n-3 polinezasićenih masnih kiselina, *trans*-kiselina i konjugirane linolne kiseline u ovčjem mlijeku

Sažetak

Izdvojene su tri skupine od po 10 ovaca sa Sardinije između 3. i 6. mjeseca laktacije kako bi se utvrdio utjecaj dodatne ishrane s n-3 masnim kiselinama na proizvodnju mlijeka i sastav masnoća u mlijeku. Kontrolna skupina (A) hranjena je smjesom sijena od *Gramineae* s kuglicama lucerne i koncentratom; ostale skupine (B i C) hranjene su kao i kontrolna, ali uz sve veću količinu ribljeg ulja. Plinskom kromatografijom određene su u mlijeku nutritivno važne masne kiseline (n-3 polinezasićene masne kiseline, PUFA; *trans*--kiseline i konjugirana linolna kiselina, CLA). Za razliku od kravljeg mlijeka, mlijeko normalno hranjenih ovaca sadržavalo je primjetne udjele n-3 PUFA i veće količine CLA. Dodatak ribljeg ulja očitovao se u povećanoj količini n-3 PUFA, CLA i *trans*-izomera u mliječnoj masti. U ovčjem mlijeku postoji pozitivna korelacija (R^2 = 0,964) između količine *trans*- i n-3 masnih kiselina, a uska pozitivna korelacija (R^2 = 0,996) između udjela CLA i *trans*-monoenskih masnih kiselina. Dodatak PUFA uzrokovao je slabo, ali statistički nesignifikantno, snizivanje postotka mliječne masti. Dijetalno riblje ulje značajno je utjecalo na proizvodnju mlijeka: ovce hranjene dodatnim n-3 masnim kiselinama proizvele su više mlijeka od onih koje su bile u kontrolnoj skupini.