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Sheep cheese naturally enriched in α-linolenic, conjugated linoleic and vaccenic acids improves the lipid profile and reduces anandamide in the plasma of hypercholesterolaemic subjects

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Abstract

Intake of dairy fat has long been considered as a risk factor for CVD. Pasture and dietary lipid supplementation have been reported to be reliable strategies in ruminant nutrition, in order to increase the content of α-linolenic acid (ALA), conjugated linoleic acid (CLA) and vaccenic acid (VA), and decrease SFA in milk fat. In the present study, we aimed at verifying whether consumption of a sheep cheese, naturally enriched in ALA, CLA and VA, would modify the plasma lipid and endocannabinoid profiles in mildly hypercholesterolaemic subjects. A total of forty-two adult volunteers (nineteen males and twenty-three females) with diagnosed mildly hypercholesterolaemia (total cholesterol 5·68–7·49 mmol/l) were randomly assigned to eat 90 g/d of a control or enriched cheese for 3 weeks, with a cross-over after 3 weeks of washout. Plasma lipids, endocannabinoids, adipokines and inflammatory markers were measured. The intake of enriched cheese significantly increased the plasma concentrations of CLA, VA, the *n*-3 fatty acids ALA and EPA, and more remarkably decreased that of the endocannabinoid anandamide. LDL-cholesterol decreased significantly (7 %). No changes were detected in the levels of inflammatory markers; however, a significant correlation was found between the plasma levels of anandamide and leptin. The control cheese modified none of the parameters measured. The results obtained do not support the view that intake of dairy fat is detrimental to hypercholesterolaemic subjects. Indeed, they show that a naturally enriched cheese possesses beneficial properties, since it ameliorates the plasma lipid profile, and more remarkably reduces endocannabinoid biosynthesis.

Key words: Endocannabinoids: Conjugated linoleic acid: Vaccenic acid: α-Linolenic acid: Dairy fat: Hypercholesterolaemia

Diets high in SFA have been associated with CVD[1–5]. Peculiarly, replacement of SFA with dietary PUFA, but not with carbohydrates or MUFA[6], decreases the risk of cardiovascular events, even though the ideal type of unsaturated fat is unclear[7]. Replacement of 5 % of energy of SFA with PUFA lowers the risk by 10 %[5]. The question is whether the effect is due to a decreasing SFA *per se*[8] or to an increase in dietary PUFA[5]. Actually, also dietary PUFA can have a strong impact on the risk of CVD, particularly when they increase the tissue *n*-6:*n*-3 highly polyunsaturated fatty acid (HPUFA) ratio with associated circulating levels of endothelial growth factor[9] and pro-inflammatory markers[10].

All these effects could be ascribed to modulations of the biosynthesis of eicosanoids and endocannabinoids, which are both strongly influenced by the tissue *n*-6:*n*-3 HPUFA ratio[11]. Down-regulation of the endocannabinoid system is also known to greatly improve several parameters of the metabolic syndrome[12]. It emerges that the effect of dietary fatty acids on the incidence of CVD is mediated by their influence on cholesterolaemia, inflammatory response and endocannabinoid tone.

The composition of dairy fat is strongly influenced by the ruminant's diet. In the latter, linoleic acid (LA) and α-linolenic acid (ALA) are the main fatty acids that are mostly modified

Abbreviations: AEA, anandamide; ALA, α-linolenic acid; CLA, conjugated linoleic acid; HPUFA, highly polyunsaturated fatty acid; LA, linoleic acid; VA, vaccenic acid.

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by anaerobic bacteria in the rumen via a biohydrogenation process aimed to produce SFA. Intermediate products of this process, such as vaccenic acid (VA) and conjugated LA (CLA), can escape the rumen and be incorporated in the ruminant's tissues and milk[13]. Fractions of LA and ALA, not modified in the rumen, are also deposited in tissues and milk[13]. All these fatty acids have been shown to be present in dairy products, at levels reflecting those in the ruminant's diet[14,15]. Among them, CLA[16], VA[17,18] and ALA[16] have been shown to possess peculiar nutritional properties as modifiers of cardiovascular risk.

Pasture and lipid supplementation of the ruminant's diet have been reported to be reliable dietary strategies in ruminant nutrition, mainly in small ruminants, in order to increase up to 4-fold the content in the milk fat of *n*-3 fatty acids, CLA and VA, and decrease up to 23 % that of SFA[15,19]. Thus, the nutritional impact of dairy fat may greatly vary due to its fatty acid composition, rendering quite difficult to make general assumptions of their nutritional properties. This is probably the reason why recent studies provided contrasting results about an inverse association between the intake of dairy products and CVD risk, varying from practically null[20–23] to small[24–26]. In studies carried out with CLA-enriched dairy products on healthy subjects, no changes in CVD risk were detected[27,28], and significant reductions of inflammatory markers, such as IL-6, IL-8 and TNF-α, were found[29]. No studies on the impact of enriched dairy products on the plasma lipid profile and endocannabinoids in hypercholesterolaemic patients have so far been performed. For this reason, we undertook the present study.

Materials and methods

Subjects

A total of forty-two adult volunteers (nineteen males and twenty-three females) with diagnosed mild hypercholesterolaemia (total cholesterol 5·68–7·49 mmol/l), 30–60 years of age, were recruited at the Center for Metabolic Diseases of the Brotzu Hospital, Cagliari, Italy. Pregnant (or those planning to become pregnant during the study period) and lactating women were excluded; also individuals with a self-reported history of diabetes, inflammatory bowel disease, pancreatitis, gallbladder or biliary disease in the past 12 months, and lactose intolerance before the screening visit were excluded. In addition, those with a history of cancer (except non-melanoma skin cancer) in the 2 years before screening, or of any major trauma or surgical event within 3 months before screening, were not enrolled. Volunteers with the following characteristics were also excluded: total cholesterol ≥7·75 mmol/l; serum TAG ≥2·82 mmol/l or ≤2·26 mmol/l; HDL ≥1·81 mmol/l; BMI ≥30 kg/m^2; or uncontrolled hypertension (systolic blood pressure ≥160 mmHg or diastolic blood pressure ≥100 mmHg) at screening. Use of lipid-altering medications or supplements, and of anticoagulants, during the 2 weeks before screening and throughout the study was prohibited. Furthermore, we selected subjects with an apoE aplotype 3/3, the most common in Sardinia, in order to avoid any variability in cholesterolaemia due to a different dietary response[30–32]. ApoE determination was carried out as described in Olivieri *et al.*[33].

Table 1. Percentage and amount (g) of the major fatty acids in 90 g of either the control cheese (CTRL) or enriched cheese (ENCH)

	Cheese fat (%)		90 g of cheese	
Fatty acids	CTRL	ENCH	CTRL	ENCH
Total SFA	59·3	45·9	13·6	10·0
Short chain (*c*4–*c*10)	16·6	11·3	3·8	2·5
*c*12 : 0	2·9	1·8	0·7	0·4
*c*14 : 0	8·5	6·1	1·9	1·3
*c*16 : 0	20·5	16·0	4·7	3·5
*c*18 : 0	10·5	10·5	2·4	2·3
Total *cis*-MUFA	19·0	21·2	4·3	4·6
*c*16 : 1*n*-9	0·3	0·3	0·1	0·1
*c*18 : 1*n*-9	18·6	20·9	4·3	4·5
Total *trans*-MUFA	3·4	10·6	0·8	2·3
*c*18 : 1-*t*11 (VA)	1·7	6·3	0·4	1·4
Total *n*-6 PUFA	2·3	2·3	0·5	0·5
*c*18 : 2*n*-6 (LA)	2·2	2·2	0·5	0·5
Total *n*-3 PUFA	0·6	2·1	0·1	0·5
*c*18 : 3*n*-3 (ALA)	0·6	2·1	0·1	0·5
Total *trans*-PUFA	0·4	1·6	0·1	0·3
Total CLA	1·0	2·8	0·2	0·6
*c*9,*t*11-CLA	0·8	2·5	0·2	0·5

VA, vaccenic acid; LA, linoleic acid; ALA, α-linolenic acid; CLA, conjugated linoleic acid.

The control and enriched cheese

The control and enriched sheep cheeses were both obtained as described previously[19]. Fat (about 26 %), protein and carbohydrate content did not differ significantly between the control and enriched sheep cheese[19]. The assigned daily portions were previously prepared. The daily portions, 45 or 90 g

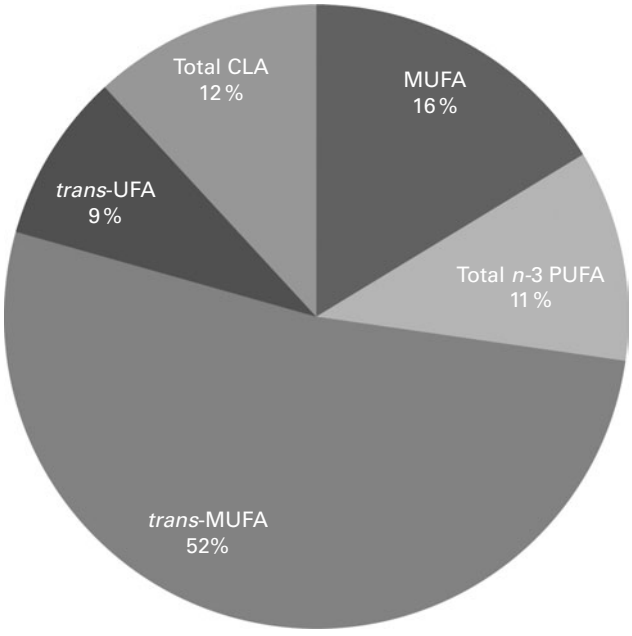

Fig. 1. Pie chart showing the percentage of increase of fatty acid classes in enriched cheese which replace SFA. UFA, unsaturated fatty acid; CLA, conjugated linoleic acid.

Table 2. Concentration of plasma fatty acids at baseline (BASE) and after an intake of 45 g/d of the control cheese (CTRL) or enriched cheese (ENCH)

(Mean values with their standard errors)

	BASE		CTRL (45 g/d)		ENCH (45 g/d)	
Fatty acids (nmol/ml plasma)	Mean	SE	Mean	SE	Mean	SE
*c*18 : 2*n*-6	3006·95	136·56	2891·25	143·72	2906·45	136·56
*c*18 : 3*n*-6	65·17	4·09	71·00	5·13	65·17	4·09
*c*20 : 3*n*-6	165·43	8·26	193·58	10·95	165·43	8·26
*c*20 : 4*n*-6	788·44	37·06	782·19	30·10	798·44	37·06
*c*22 : 4*n*-6	14·89	0·85	17·92	1·39	14·89	0·85
*c*18 : 3*n*-3	43·02	2·13	40·31	3·79	42·02	2·13
*c*20 : 5*n*-3	83·46	6·87	87·12	7·51	83·46	6·87
*c*22 : 6*n*-3	183·38	10·56	189·63	8·68	188·38	10·56
*c*18 : 1*n*-9	2570·87	104·00	2448·02	113·33	2519·51	114·49
*c*20 : 3n9	14·32	1·33	16·88	1·21	12·32	1·02
*c*14 : 0	139·47	9·12	149·57	14·33	124·47	10·82
*c*15 : 0	28·51	1·54	28·48	2·23	23·51	1·49
*c*16 : 0	3078·05	155·68	2904·08	172·61	2978·05	155·68
*c*18 : 0	737·59	33·84	780·99	32·99	787·59	33·84
*c*9,*t*11-CLA	16·97[a]	0·50	17·87[a]	0·66	26·62[b]	0·80
VA	16·12	1·35	15·85	1·61	12·68	1·01

CLA, conjugated linoleic acid; VA, vaccenic acid.
[a,b] Mean values with unlike superscript letters were significantly different ($P<0·05$).

of the edible part (no crust), were sealed under vacuum and stored at −20°C. The subjects were instructed to consume the given portion per d preferably during meals, for 3 weeks. Dietary monitoring revealed that dietary cheese replaced other foods without changing total energy and macronutrient distribution intake.

Design of the main study

The present study was a 3-week, randomised, single-blind, controlled, cross-over clinical trial, conducted at the State Hospital Brotzu in Cagliari, Italy. The subjects were randomly assigned to eat 90 g/d of the control or enriched cheese for 3 weeks, with a cross-over after 3 weeks of washout. The study included five visits: two screening/baseline visits at weeks −1 and 0; one end of intake of 90 g/d of cheese visit at week 3; one end of the first washout visit at week 6; one end of treatment after crossing over at week 9. This procedure was subsequently repeated with a cheese intake of 45 g/d. In each visit, subjects were evaluated for clinical and anthropometric parameters (height, weight and girth). Venous blood samples were collected from the antecubital vein into evacuated plastic tubes (Vacutainer) without anticoagulant, after an overnight fast, and centrifuged at 2000 ***g*** for 10 min at 4°C. At the first screening, subjects were trained to keep a food diary for monitoring their food intake during the protocol time.

Table 3. Concentration of plasma fatty acids at baseline (BASE) and after an intake of 90 g/d of the control cheese (CTRL) or enriched cheese (ENCH)

(Mean values with their standard errors)

	BASE		CTRL (90 g/d)		ENCH (90 g/d)	
Fatty acids (nmol/ml plasma)	Mean	SE	Mean	SE	Mean	SE
*c*18 : 2*n*-6	3218·22	104·84	3228·98	119·37	3089·09	108·05
*c*18 : 3*n*-6	76·00	4·88	78·72	4·96	69·92	4·33
*c*20 : 3*n*-6	198·08	6·84	204·25	9·68	185·11	8·88
*c*20 : 4*n*-6	860·10	23·56	877·54	20·42	825·07	21·84
*c*22 : 4*n*-6	17·38	0·88	18·98	1·18	15·90	0·95
*c*18 : 3*n*-3	41·62[a]	2·21	47·52[a,b]	4·36	56·58[b]	3·39
*c*20 : 5*n*-3	81·05[a]	5·70	95·54[a,b]	6·80	110·63[b]	7·78
*c*22 : 6*n*-3	195·83	9·06	213·20	12·78	217·75	10·09
*c*18 : 1*n*-9	2657·35	104·00	2613·78	102·56	2525·83	106·95
*c*20 : 3n9	14·72	1·23	18·25	1·29	14·57	1·14
*c*14 : 0	149·17	17·02	165·00	18·06	160·79	17·51
*c*15 : 0	28·93[a]	1·86	35·38[a,b]	2·07	37·77[b]	2·39
*c*16 : 0	3100·14	154·93	3170·03	162·72	3246·33	157·85
*c*18 : 0	793·70	30·46	739·77	25·28	836·48	29·53
*c*9,*t*11-CLA	15·37[a]	0·50	27·94[b]	0·88	38·75[c]	1·06
VA	14·42[a]	1·35	14·62[a]	1·77	31·44[b]	2·59

CLA, conjugated linoleic acid; VA, vaccenic acid.
[a,b,c] Mean values with unlike superscript letters were significantly different ($P<0·05$).

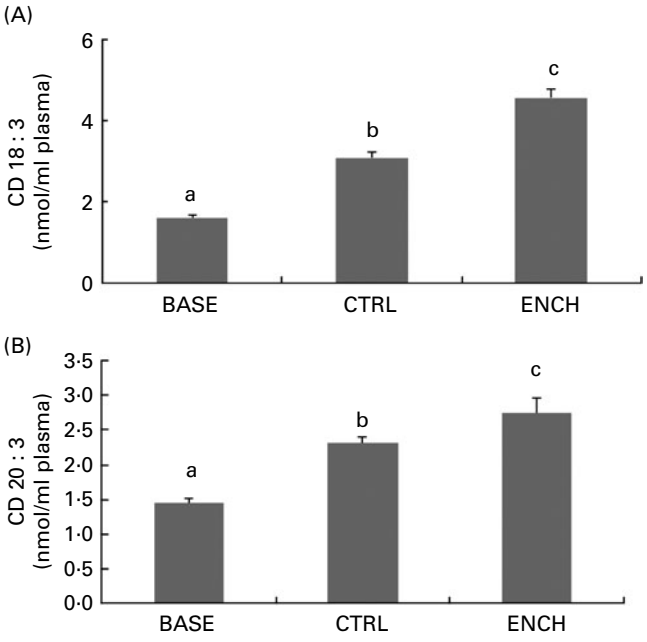

Fig. 2. Plasma concentrations of the conjugated linoleic acid metabolites (A) conjugated linolenic acid (CD 18 : 3; *cis*-6,*cis*-9,*trans*-11-18 : 3) and (B) conjugated eicosatrienoic acid (CD 20 : 3; *cis*-8,*cis*-11,*trans*-13-20 : 3), at baseline (BASE) and after consumption of 90 g/d of the control cheese (CTRL) or enriched cheese (ENCH). Values are means, with their standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$).

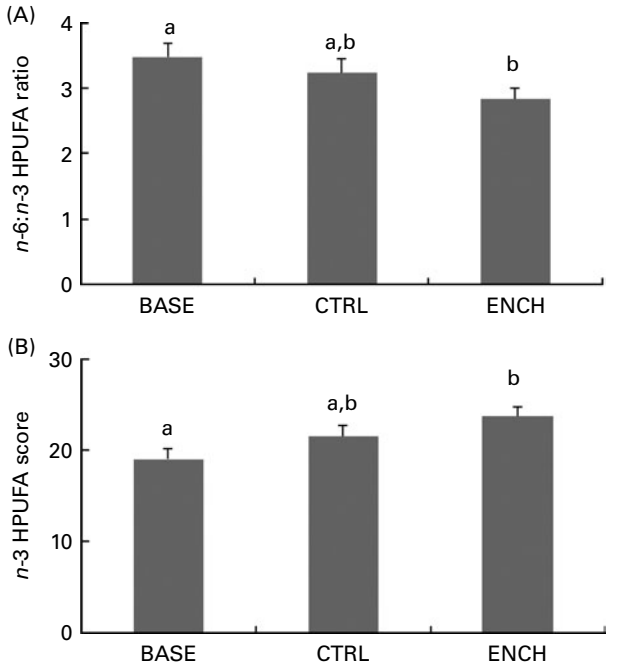

Fig. 3. (A) Plasma *n*-6:*n*-3 highly polyunsaturated fatty acid (HPUFA) ratio and (B) *n*-3 HPUFA score at baseline (BASE) and after consumption of 90 g/d of the control cheese (CTRL) or enriched cheese (ENCH). Values are means, with their standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the ethical committee of the Brotzu Institution. This trial was registered at ClinicalTrials.gov (identifier no. NCT01570270). Written informed consent was obtained from all subjects.

Study with synthetic conjugated linoleic acid

CLA pills were supplied by Lipid Nutrition B.V., Wormerveer, Netherlands, and contained 1 g of 80 % CLA (63 % *c*9*t*11, 13 % *t*10,*c*12, 3 % *c*11,*t*13), 14 % oleic acid, 3 % LA and 5 % palmitic acid. Pills containing 1 g of a palm oil–soyabean oil mix (1:1) were used as the control.

At baseline, twenty subjects who had already participated in the previous main study were randomly assigned in a single blind to receive a daily pill of either CLA or the control. The subjects were instructed to take the capsules preferably during meals, for 3 weeks.

Pills containing 0·8 g CLA were chosen because this dose is close to its daily intake in the previous trial study. This is evinced by summing the CLA content in 90 g of cheese (Table 1), with its amount generated by a 20 % conversion of the VA content (Table 1) via Δ-9 desaturation[34,35].

Lipid analyses

Total lipids were extracted from plasma using chloroform–methanol (2:1, v/v)[36]. Aliquots were mildly saponified, as described previously[37], to obtain NEFA for HPLC analysis. Separation of fatty acids was carried out with an Agilent 1100 HPLC system (Agilent) equipped with a diode array detector as reported previously[38]. Conjugated diene unsaturated fatty acids, including fatty acid hydroperoxides, were detected at 234 nm. Spectra (195–315 nm) of the eluate were obtained every 1·28 s and were electronically stored. Second-derivative UV spectra of the conjugated diene fatty acids were generated using Phoenix 3D Agilent Chemstation software (Agilent). These spectra were taken to confirm the identification of the HPLC peaks[39]. Details of the methodology regarding the characterisation of conjugated diene unsaturated fatty acids in both reference and biological samples have been published[38]. Measurements of SFA and further confirmation of *trans*-monoenes and CLA isomers were

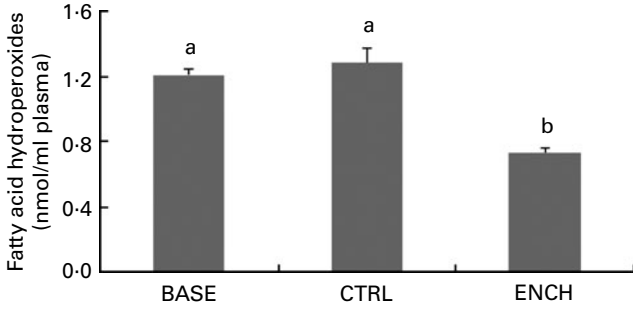

Fig. 4. Concentrations of plasma fatty acid hydroperoxides at baseline (BASE) and after consumption of 90 g/d of the control cheese (CTRL) or enriched cheese (ENCH). Values are means, with their standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

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Table 4. Anthropometric characteristics and plasma lipid levels at baseline (BASE) and after an intake of 45 g/d of the control cheese (CTRL) or enriched cheese (ENCH)
(Mean values with their standard errors)

	BASE		CTRL (45 g/d)		ENCH (45 g/d)	
	Mean	SE	Mean	SE	Mean	SE
BMI (kg/m^2)	26·65	0·51	26·49	0·57	26·32	0·59
Male waist circumference (cm)	89·68	1·01	88·93	1·03	89·00	1·00
Female waist circumference (cm)	82·95	1·65	82·59	1·58	81·64	1·60
Total cholesterol (mmol/l)	6·26	0·10	6·49	0·11	6·39	0·11
LDL-C (mmol/l)	4·15	0·12	4·32	0·11	4·17	0·08
HDL-C (mmol/l)	1·43	0·04	1·54	0·04	1·42	0·03
Non-HDL-C (mmol/l)	4·83	0·11	4·95	0·10	4·95	0·11
Total cholesterol:HDL-C	4·51	0·14	4·34	0·13	4·62	0·14
TAG (mmol/l)	1·35	0·13	1·39	0·12	1·43	0·13

LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; non-HDL-C, non-HDL-cholesterol.

obtained by GC and silver ion HPLC as described in Cappa *et al.*[40] and Ip *et al.*[41].

Determination of anandamide (AEA) and 2-arachidonoylglycerol, the HPUFA *n*-6:*n*-3 ratio and the *n*-3 HPUFA score were performed as described in Piscitelli *et al.*[42] and Stark[43].

Analyses of adipokines and cytokines

Serum leptin, adiponectin, IL-6 and C-reactive protein were determined by sandwich ELISA tests (Abcam) as described previously[44]. The ranges of the assays were as follows: 15·6–1000 pg/ml for leptin; 1·0–100 ng/ml for adiponectin; 0·16–10 pg/ml for IL-6; 78–5000 pg/ml for C-reactive protein.

Clinical chemistry analyses

Whole venous blood was also collected in tubes without anticoagulant and centrifuged at 2000 ***g*** for 10 min at 4°C, and aliquots of the supernatants were stored at − 80°C until assayed. Lipid and biochemical variables were assessed by conventional methods and performed at a clinical chemistry hospital laboratory.

Statistical analyses

One-way ANOVA with the Tukey test for *post hoc* analyses was applied to evaluate statistical differences between groups. Correlation between leptin and AEA was assessed by Pearson's simple correlation analysis. P values $<0·05$ were considered as significant.

Results

Clinical trial

Fatty acid profile of the control and enriched cheese. Table 1 shows the percentage content of major fatty acids in the control and enriched cheese consumed by the volunteers. With respect to the control, the enriched cheese has 3, 3·5 and 4 times higher levels of CLA, ALA and VA, respectively. To these increases corresponds a 23 % decrease in SFA. Interestingly, as shown in Fig. 1, in the enriched cheese, SFA were mainly replaced by *trans*-MUFA, of which VA represented about 60 %.

Plasma fatty acid profile in subjects on the control or enriched cheese. Tables 2 and 3 show major fatty acids

Table 5. Anthropometric characteristics and plasma lipid levels at baseline (BASE) and after an intake of 90 g/d of the control cheese (CTRL) or enriched cheese (ENCH)
(Mean values with their standard errors)

	BASE		CTRL (90 g/d)		ENCH (90 g/d)	
	Mean	SE	Mean	SE	Mean	SE
BMI (kg/m^2)	26·64	0·44	26·30	0·49	26·03	0·49
Male waist circumference (cm)	90·13	0·88	90·21	1·22	89·58	0·91
Female waist circumference (cm)	83·89	1·41	84·55	1·52	83·66	1·30
Total cholesterol (mmol/l)	6·29^a	0·07	6·62^a	0·13	5·96^b	0·07
LDL-C (mmol/l)	4·29^a	0·06	4·41^a	0·10	4·00^b	0·05
HDL-C (mmol/l)	1·43^a	0·04	1·60^b	0·05	1·44^a	0·02
Non-HDL-C (mmol/l)	4·86^a	0·08	4·88^{a,b}	0·17	4·50^b	0·07
Total cholesterol:HDL-C	4·54	0·15	4·25	0·12	4·23	0·14
TAG (mmol/l)	1·26	0·09	1·36	0·15	1·26	0·12

LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; non-HDL-C, non-HDL-cholesterol.
^{a,b} Mean values with unlike superscript letters were significantly different ($P<0·05$).

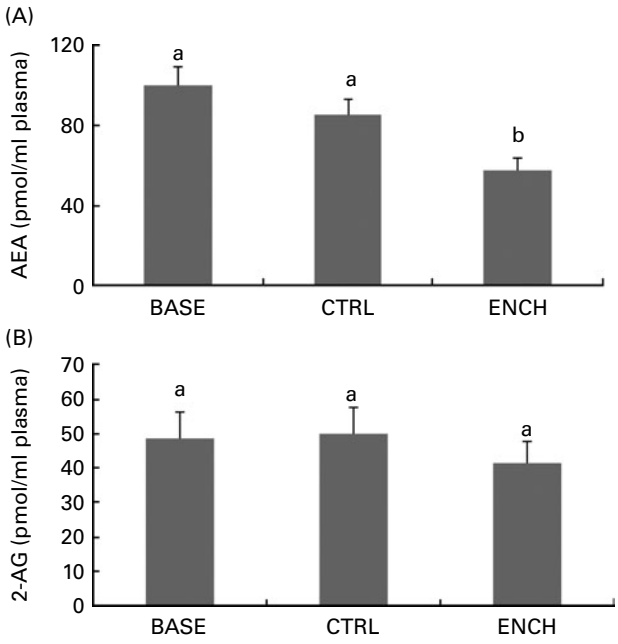

Fig. 5. Plasma concentrations of endocannabinoids: (A) anandamide (AEA) and (B) 2-arachidonoylglycerol (2-AG) at baseline (BASE) and after consumption of 90 g/d of the control cheese (CTRL) or enriched cheese (ENCH). Values are means, with their standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

detected in the plasma of subjects at baseline and after the intake of 45 or 90 g/d, respectively, of both the control and enriched cheese. No significant changes were found for most of the concentrated fatty acids such as oleic, palmitic, linoleic and arachidonic acids. Pentadecanoic acid, a marker of dairy fat intake[45], was found to be significantly increased after the 90 g/d intake of both control and enriched cheese, while the intake of 45 g/d did not modify its level.

Consumption of the enriched cheese resulted in concentrations of CLA and VA significantly higher than those after consumption of the control cheese, and higher as well of the baseline level, as did conjugated linolenic acid (*cis*-6,*cis*-9,*trans*-11-18 : 3) and conjugated eicosatrienoic acid (*cis*-8,*cis*-11,*trans*-13-20 : 3), the metabolites of CLA (Fig. 2). Interestingly, the 45 g/d intake of the enriched cheese resulted in a CLA concentration similar to that after the 90 g/d intake of control cheese. Plasma ALA and EPA also increased significantly with respect to baseline after the 90 g/d intake of enriched cheese. In addition, the EPA increase contributed to a significant decrease in the *n*-6:*n*-3 HPUFA ratio (Fig. 3(A)), and to an increase in the *n*-3 HPUFA score (Fig. 3(B)), which is a reliable biomarker of the *n*-3 HPUFA status in tissues[43]. Interestingly, plasma fatty acid hydroperoxides decreased significantly despite the increase in HPUFA (Fig. 4).

BMI and clinical chemistry analyses. As shown in Table 4, consumption of 45 g/d of the control or enriched cheese did not significantly change BMI, waist circumference and all the lipid parameters taken into consideration. However, as shown in Table 5, the intake of 90 g/d of the enriched cheese, while not modifying BMI and waist circumference, decreased significantly the level of total, LDL- and non-HDL-cholesterol (54 % of the subjects showed a decrease higher than 5 % with an average of 15 % decrease). The 90 g/d intake of control cheese increased HDL-cholesterol significantly, whereas LDL- and total cholesterol increased but not significantly. No significant changes were detected for glycaemia, creatinine and uricaemia with any of the treatments (data not shown).

Endocannabinoids, adipokines and inflammatory marker analyses. Analyses of endocannabinoids showed a strong and significant decrease in AEA (about 40 %) with respect to both baseline and control cheese treatment after the 90 g/d intake of enriched cheese, while 2-arachidonoylglycerol decreased but not significantly (Fig. 5).

In order to verify whether the AEA decrease was associated with adipokines and inflammatory markers, we measured the levels of leptin, adiponectin, IL-6 and C-reactive protein in sixteen subjects (eight males and eight females) at the beginning and end of each experimental period. None of these parameters changed significantly (data not shown). Interestingly, after treatment with 90 g/d of enriched cheese, leptin and AEA concentrations were strongly correlated (Fig. 6).

The 45 g/d intake of enriched cheese did not modify the endocannabinoid profiles when compared with either the baseline values or after the 45 g/d intake of control cheese (data not shown).

Study with synthetic conjugated linoleic acid

The 0·8 g/d intake of synthetic CLA resulted in plasma levels of CLA and its metabolites similar to those obtained with 90 g/d of enriched cheese (Fig. 7), but neither the plasma lipid profile nor the levels of endocannabinoids changed significantly (data not shown).

Discussion

In the present study, we have shown that an intake of 90 g/d of an enriched cheese resulted in significant increases in the plasma concentrations of CLA, VA and ALA, increases reflecting their high content in the cheese. The concentration of

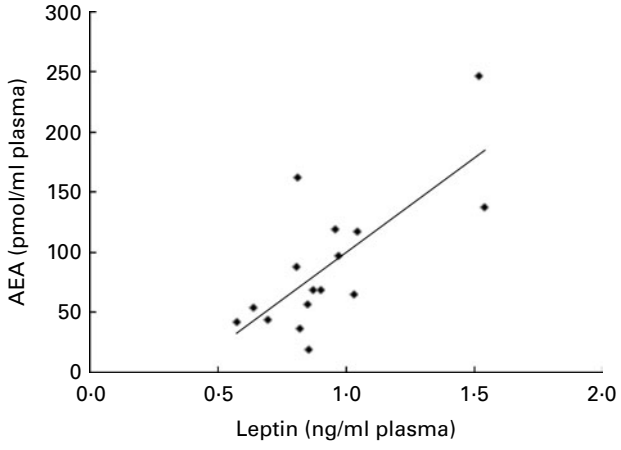

Fig. 6. Correlation between the plasma concentrations of anandamide (AEA) and leptin after consumption of 90 g/d of enriched cheese. R^2 0·53, $P<0·05$.

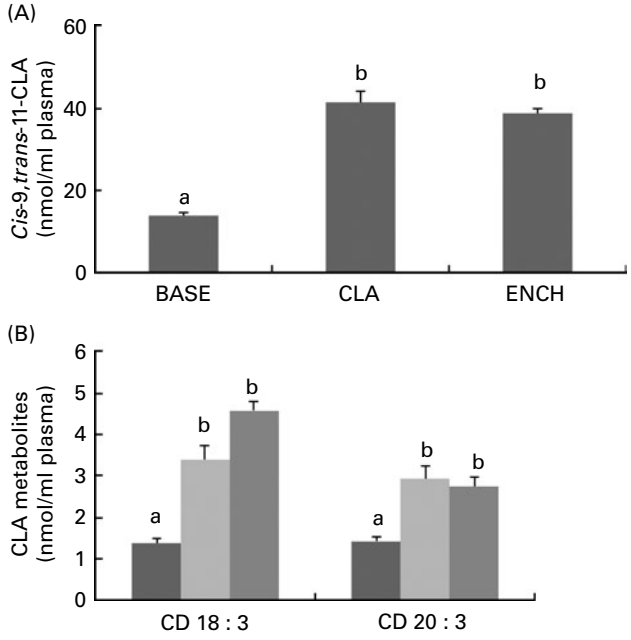

Fig. 7. (A) Conjugated linoleic acid (CLA) plasma levels at baseline (BASE), after intake of 0·8 g synthetic CLA per d and after intake of 90 g enriched cheese per d (ENCH), (B) conjugated diene (CD) 18:3 and CD 20:3 plasma levels at baseline (■), after intake of 0·8 g synthetic CLA per d (■) and after intake of 90 g enriched cheese per d (■). Values are means, with their standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

EPA also increased, indicating a chain elongation and desaturation of ALA to EPA, and a contribution to an overall increase in the *n*-3 HPUFA score along with a decrease in the *n*-6:*n*-3 HPUFA ratio. In recent studies, we have shown that a lower *n*-6:*n*-3 ratio results in a decrease of endocannabinoids in different tissues of rats[46], mice[42] and human plasma[47]. Interestingly, AEA was strongly correlated with leptin concentration only after the enriched cheese phase with 90 g/d. This finding confirms previous evidence of an association between AEA and adiposity[48]. However, given the experimental conditions of the present study, it is not possible to establish whether a decrease in AEA influences adiposity, and thereby leptin levels, or the other way around, a leptin decrease determines the AEA reduction. Moreover, a 3-week treatment is probably not sufficient to detect changes in adiposity, in particular changes in endocannabinoid system homeostasis. Indeed, even a direct antagonism of cannabinoid receptor 1 by rimonabant (20 mg/d), in obese subjects, required 2–3 months to start reducing the body weight, and ameliorating dyslipidaemia and insulin resistance[49].

Whether CLA and/or VA are able to influence endocannabinoid biosynthesis is not clear as yet. In one paper[50], changes in endocannabinoid levels, only in the brain, have been reported when rats were treated with high doses of CLA. In the present study, the intake of 0·8 g/d of synthetic CLA did not modify the plasma concentration of endocannabinoids, even though the plasma levels of CLA and its metabolites were similar to those found after the enriched cheese treatment. These results also demonstrate that the intake of 90 g/d of the enriched cheese does amount into about 0·8 g/d intake of CLA. Indeed, with the predicted intake of CLA with enriched cheese being about 0·6 g/d, the conversion of VA to CLA must be about 20 %, as shown previously[34,35]. Moreover, given that the intake of 0·8 g/d of synthetic CLA did not modify endocannabinoid and plasma cholesterol levels, the changes detected with enriched cheese cannot be attributed only to CLA and/or its metabolites. It cannot be ruled out, however, that higher doses of CLA and/or a synergistic effect with VA may affect the biosynthesis of endocannabinoids.

From data in the literature, no single component found to be increased in the plasma of subjects treated with the enriched cheese appeared to clearly affect the level of LDL-cholesterol. Indeed, no change in LDL-cholesterol was observed in human subjects after ALA[51] or CLA[52] supplementation.

A blockade of the cannabinoid receptor affected lipid metabolism, but not the levels of LDL- or total cholesterol[53]. However, a recent study[54] has shown that the hypolipidaemic effects of chronic *c*9,*t*11-CLA supplementation on circulating dyslipidaemia were enhanced by the addition of VA in the JCR:LA-cp rat, a model of the metabolic syndrome. It is therefore likely that a concerted action of all these components, along with a 23 % decrease in SFA, may have resulted in the decrease of LDL-cholesterol seen in the present study, even though, in the enriched cheese, SFA were mainly replaced by *trans*-MUFA and not by PUFA, a condition that should not favour a hypocholesterolaemic effect[7]. However, it has been shown that *trans*-MUFA from industrially produced and from natural sources have different effects on the plasma cholesterol profile, with a more favourable effect of those from natural sources[17].

The effect of the control cheese in increasing HDL-cholesterol may well be explained by the effect of SFA which are known to increase both HDL- and LDL-cholesterol[55]. In the present study, however, the increase in LDL-cholesterol was not significant.

Interestingly, beneficial properties of the enriched cheese were obtained with a 90 g/d and not with a 45 g/d intake, suggesting a dose-dependent effect, at least after a 3-week treatment. Further studies using longer treatments may better evaluate the dose–time relationship. In addition, dietary records and levels of pentadecanoic acid indicated the average cheese intake of 45 g/d in these subjects, which is in agreement with a previous study[56] that reported an average cheese intake in south Italy between 40 and 50 g/d.

It has been claimed that increases in HPUFA may lead to an increased susceptibility of lipid peroxidation[57,58]. However, the present results show that intake of the enriched cheese significantly decreased the plasma level of fatty acid hydroperoxides, which is in contrast also with reports suggesting that dietary CLA triggers lipid peroxidation[59]. The last notion, furthermore, failed to gain support from a more recent study, which suggested that increases in isoprostane, a marker of lipid peroxidation, after CLA intake, might be due to an impairment of its catabolism[60].

Perturbations of plasma and probably tissue fatty acid profiles, in favour of *n*-3 PUFA, may decrease the susceptibility to inflammation[11], even if changes in IL-6 and C-reactive protein were not detected in the present study, probably because baseline levels of cytokines were in physiological ranges. Recently, it has been reported that a CLA-enriched cheese was able to significantly reduce inflammatory markers such as IL-6, IL-8 and TNF-α[29]. That study was carried out for 10 weeks on healthy subjects. It seems likely therefore that the apparent discrepancy in the present study may be due to the length of treatment, and/or the physiopathological characteristics of the subjects under study. More focused studies should thus be carried out to explore the possible role of an intake of enriched cheese on oxidative stress and inflammatory parameters.

In conclusion, it emerged from the present study that a cheese with a peculiar fatty acid profile influences plasma lipids, and more remarkably endocannabinoid biosynthesis. To our knowledge, this is the first report showing that intake of a food is able to affect the endocannabinoid system. This capacity may have important implications on its ability to modify parameters of the metabolic syndrome.

The major limitation of the present study was the relatively short period of treatment. However, giving 90 g/d of cheese to hypercholesterolaemic subjects for longer periods would not have been advisable, due to its high content of saturated fat[5]. Nonetheless, the present data cast doubts on the general assumption that dairy fat, *per se*, is detrimental to hypercholesterolaemic subjects, indicating that nutritional assessments of products of animal origin cannot be accurate, without taking into consideration their detailed composition, since the latter is affected by the animal diet.

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