Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 22 (2011) 495–501
Journal of Nutritional Biochemistry

# Enhancement of antibody synthesis in rats by feeding *cis*-9,*trans*-11 conjugated linoleic acid during early life☆

Carolina Ramírez-Santana[a,b,c], Cristina Castellote[a,b,c], Margarida Castell[a,b], Carolina Moltó-Puigmartí[c,d], Montserrat Rivero[e], Francisco J. Pérez-Cano[a,b,*], Àngels Franch[a,b,c]

[a]*Department of Physiology, Faculty of Pharmacy, University of Barcelona, E-08028 Barcelona, Spain*
[b]*Institut de Recerca en Nutrició i Seguretat Alimentària (INSA-UB), E-08028 Barcelona, Spain*
[c]*CIBER Epidemiología y Salud Pública (CIBERESP), E-08003 Spain*
[d]*Department of Nutrition and Food Science, Faculty of Pharmacy, University of Barcelona, E-08028 Barcelona, Spain*
[e]*Ordesa Group, Research Department, Science Park of Barcelona, E-08028 Barcelona, Spain*

Received 12 February 2010; received in revised form 30 March 2010; accepted 7 April 2010

**Abstract**

Previous studies have demonstrated that the intake of a 1% conjugated linoleic acid (CLA) diet in an 80:20 mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 exerts age-specific effects on the immune system: immunoglobulin enhancement and proliferative down-modulation in neonatal and adult rats, respectively. The present study evaluates the influence of the same diet on antibody synthesis of early infant Wistar rats during suckling and/or after weaning. Dietary supplementation was performed during suckling and early infancy (4 weeks), only during suckling (3 weeks), or only in early infancy (1 week). CLA content in plasma and serum immunoglobulin (Ig) G, IgM and IgA concentration were determined. Proliferation, cytokines and Ig production were evaluated on isolated splenocytes. *Cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA isomers were detected in the plasma of all CLA-supplemented animals, and the highest content was quantified in those rats supplemented over the longest period. These rats also exhibited higher concentrations of serum IgG, IgM and IgA. Moreover, splenocytes from CLA-supplemented rats showed the highest IgM and IgG synthesis and interleukin (IL)-6 production, whereas their proliferative ability was lower. In summary, in infant rats, we observed both the enhance antibody synthesis previously reported in neonates, and the reduced lymphoproliferation previously reported in adults.


*Keywords:* Conjugated linoleic acid; Early life; Immunoglobulin synthesis; Lymphocyte proliferation

## 1. Introduction

Historically, considerable attention has been focused on neonatal nutrition, and within this field, the importance of polyunsaturated fatty acids (PUFAs) other than arachidonic acid and docosahexaenoic acid (DHA) has been highlighted [1]. Neonatal development, particularly neonatal immunity, is influenced after birth by consumption of breast milk [2,3]. Breast milk includes many bioactive components, such as antibodies, growth factors, cytokines, nucleotides, cells and lipids as conjugated linoleic acid (CLA), that influence maturation of the developing immune system [4,5]. In addition, it has been demonstrated in animal models that milk-derived immune factors can cross the neonatal intestine and influence beyond this compartment [3]; moreover, they can provide further protection in weaning [6].

CLA was first identified as a main component of fried ground beef with anti-carcinogenic properties [7], and it has also been found in human breast milk ranging from 2.23 to 5.43 mg/g fat [8]. Many effects have been attributed to CLA, such as reducing atherosclerosis severity [9,10] and body fat, while enhancing lean body mass [11]. The diversity of the biological activities of CLA is due to its variable composition, since it is a mixture of more than 20 geometric and positional isomers. However, the primary research is focused on the two biologically active isomers of CLA: *cis*-9,*trans*-11 (*c*9,*t*11) and *trans*-10,*cis*-12 (*t*10,*c*12) [12]. These two isomers have shown additive, independent, or even antagonistic effects. Both CLA isomers have anticarcinogenic effects and probably immunomodulatory properties, but the *t*10,*c*12 CLA isomer is better known to be responsible for body fat reduction [13]. The *c*9,*t*11 isomer constitutes more than 80% of CLA in breast milk and dairy products and, because of that, its influence in early age becomes of special interest [8,14,15].

Over the past 20 years, numerous studies have been conducted in rodents, pigs and chickens, using CLA at doses ranging from 0.5% to 1% of total dietary fat [16]; however, most of them have been performed

*Abbreviations:* CLA, conjugated linoleic acid; PPAR, peroxisome proliferator-activated receptor; IL, interleukin; Ig, immunoglobulin; IFN, interferon.

☆ The authors have declared no conflict of interest.

* Corresponding author. Departament de Fisiologia, Facultat de Farmàcia, Av. Joan XXIII s/n, E-08028 Barcelona, Spain. Tel.: +34934024505; fax: +34934035901.
*E-mail address:* franciscoperez@ub.edu (F.J. Pérez-Cano).

0955-2863/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2010.04.005

in adult animals, and in humans, using 50:50 isomeric mixtures of *c*9, *t*11 and *t*10,*c*12 CLA. These mixtures and the pure isomers have reported different effects depending on the age of the animals used. CLA modulates lymphoproliferation in adult animals and in humans by decreasing polyclonal proliferative ability, like many other PUFAs, but increases specific proliferative response after challenge [17–20]. However, no effect on lymphoproliferation is observed in neonatal animals fed CLA [21]. On the other hand, although several studies in adult animals following CLA diets do not modify in vitro or in vivo immunoglobulin (Ig) production [19,20,22,23], some enhancing effects on immunoglobulin synthesis are described for neonatal and young rodents after CLA consumption [21,24,25].

In previous studies, we have demonstrated substantial immunomodulatory effects on neonatal and adult rats after a *cis*-9,*trans*-11 CLA-enriched diet. The present work evaluates the influence of the same CLA diet on early infant Wistar rats (4 weeks old). At this age, their immune system is still in maturation (i.e., antibody production); however, some immune functions are already mature and very similar to those of adult animals (i.e., lymphoproliferative ability). Moreover, as it has been suggested that CLA intake during early stages of development may have effects later in life [26,27], we have also studied whether CLA supplementation limited to suckling produces effects which can last until early infancy (1 week later).

## 2. Material and methods

### 2.1. Animals

Pregnant Wistar rats at 7 days of gestation were obtained from Harlan (Barcelona, Spain). The animals were housed in individual cages under controlled temperature and humidity conditions in a 12 h:12 h light:dark cycle and had access to food and water ad libitum. The rats were allowed to deliver at term. The delivery day was identified as Day 1 of life. Litters were randomized and unified to 10 pups per lactating dam; pups had free access to the nipples and rat diet. All daily handling was done in the same time range to avoid the influences of biological rhythms. Body weight and body length (nose–anus length) were used to determine the following morphometrical parameters: body mass index (BMI), calculated as body weight/length$^2$ (g/cm$^2$) and Lee index, calculated as $\sqrt[3]{}$weight/length ($\sqrt[3]{}$g/cm).

Twenty-eight-day-old rats were anaesthetized with ketamine/xylazine to obtain spleens and blood for plasma and serum samples, which were immediately frozen at −80°C until processing. Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation at the University of Barcelona and the Catalonian Government (CEEA 303/05 UB/DMA 3242).

### 2.2. Diets

The standard diet corresponded to the American Institute of Nutrition (AIN)-93G formulation [28], containing 7% soybean oil. A 1% CLA diet was obtained from modified standard flour AIN-513 (Harlan) containing 10 g CLA/kg [27,28]. Thus, the supplemented diet contained 6% soybean oil plus 1% CLA oil. The CLA isomer mixture was approximately 80% *c*9,*t*11 and 20% *t*10,*c*12 from the total CLA isomers in oil. This proportion has been chosen due to its resemblance to that one present in breast milk [8]. The CLA mixture had 0.69% free fatty acids as oleic acid, a peroxide value of 0.2 mEq/kg, 5.6% saturated fatty acids and less than 5% of minor CLA isomers. CLA oil was kindly supplied by Loders Croklaan, Lipid Nutrition, Wormerveer, The Netherlands.

As suckling pups did not eat pelleted diet until weaning, daily administration of 1.5 mg CLA/g per rat from Day 1 to 21 corresponds to a 1% CLA diet in suckling animals as previously described [21]. This data is based on the daily intake of rats from 21 to 28 days old (10–15 g chow/100 g of rat body weight). Low-capacity syringes (Hamilton Bonaduz, Bonaduz, Switzerland) adapted to oral 25- or 23-gauge gavage tubes, 27 mm in length (ASICO, Westmont, IL, USA), were used for oral administration before and after Day 5, respectively. To allow gastric emptying, litters were separated from dams 1 h before oral supplementation.

### 2.3. Study design

On the delivery day, animals were distributed into four experimental groups (two dams with 10 pups each, $n$=20/group), according to the total period of CLA supplementation (Fig. 1):

Four-week group: During suckling, pups received CLA daily by oral gavage; after weaning, animals were fed 1% CLA pelleted diet from Day 21 to 28. Total period of supplementation, 4 weeks.
Three-week group: During suckling, pups received CLA daily by oral gavage; after weaning, animals were fed standard diet until day 28. Total period of supplementation, 3 weeks.
One-week group: Rats received 1% CLA-pelleted diet exclusively for one week after weaning (Days 21–28). Total period of supplementation, 1 week.
Control group: these animals constitute the control diet group. Total period of supplementation, 0 week.

All animals from the four dietary groups were sacrificed at the age of 28 days.

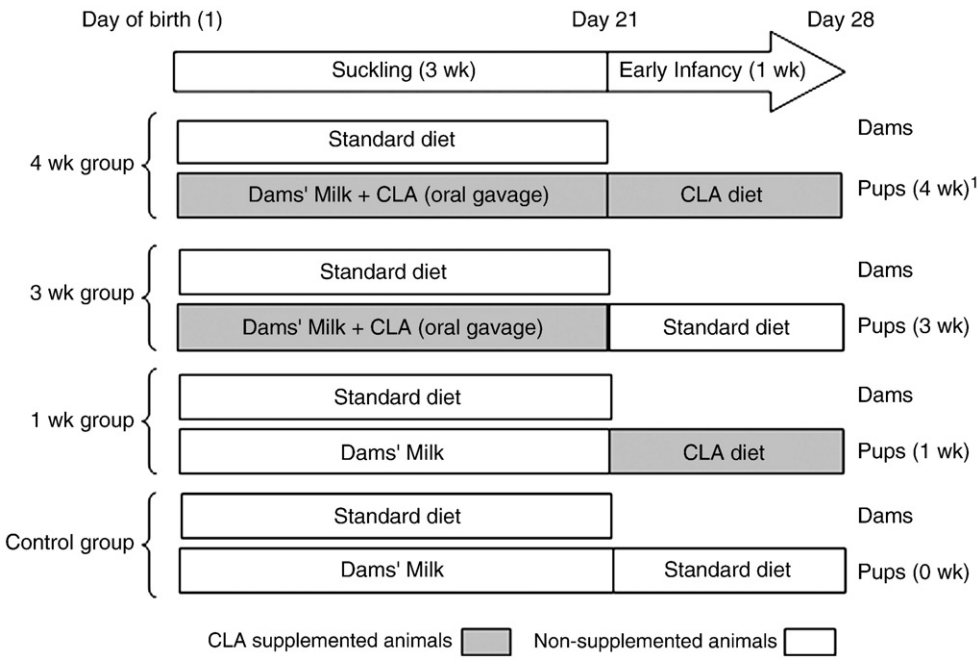


Fig. 1. Diagram of the experimental design beginning on the day of birth until day 28 of life. [1]Total period of CLA supplementation from the day of birth until 1 week after weaning.

### 2.4. CLA and fatty acid quantification in rat plasma

Fatty acid composition and *c*9,*t*11 and *t*10,*c*12 CLA isomers content were quantified in rat plasma by fast gas chromatography using a capillary column (40 m× 0.18 mm×0.20 μm), coated with RTX-2330 non-bonded stationary phase (poly 90% biscyanopropyl-10% cyanopropylphenyl) siloxane from Thames Restek UK (Saunderton, UK) as previously described [29]. Fatty acid methyl esters of the sample were identified by the comparison of their relative retention times with those of well-known fatty acid methyl esters (FAMEs) standards. Quantification was based on the amount of the internal standard recovered. The results were expressed in relative amounts (% total fatty acids).

### 2.5. Isolation of spleen lymphocytes

Spleen cell suspensions were obtained in sterile conditions as previously described [30]. Briefly, the tissue was passed through a steel mesh (Cellector; Bellco, Vertrieb, Austria). Erythrocytes were lysed from cell suspension. After washing, cells were resuspended at $10^6$ cells/ml in RPMI-10% fetal bovine serum containing 0.05 mmol/L 2-mercaptoethanol (Merck, Darmstadt, Germany), 100 IU/ml streptomycin–penicillin (Sigma, St. Louis, MO, USA) and 2 mmol/L L-glutamine (Sigma). Cell viability was determined by double staining with acridine orange and ethidium bromide (Sigma). Cells were plated and cultured in different conditions according to assay.

### 2.6. Spleen lymphocyte proliferation

Lymphocytes were cultured at $1\times10^5$ cells/100 μl in a 96-well plate and stimulated with phorbol myristate acetate (PMA) plus ionomycin (Io), both at 250 ng/ml (Sigma) at 37°C and 5% $CO_2$. Lymphocyte proliferation was determined by a modified ELISA using Cell Proliferation Biotrak (Amersham Biosciences, Munich, Germany) after 72 h of incubation as previously described [23]. The proliferation rate (%) was expressed considering the control diet group as 100%; results based on absorbance (Ab) quantification were expressed as follows:

Proliferation rate (%)=(*A*/*B*)×100, where,

$A=[(Ab_{\text{stimulated cells}}-Ab_{\text{nonstimulated cells}})/Ab_{\text{non-stimulated cells}}]_{\text{CLA diet}}$, and
$B=[(Ab_{\text{stimulated cells}}-Ab_{\text{nonstimulated cells}})/Ab_{\text{nonstimulated cells}}]_{\text{standard diet}}$.

A parallel plate was cultured with the same samples and conditions to determine the cell viability after 72 h of culture.

### 2.7. In vitro cytokine production

Splenocytes were cultured at $3\times10^6$ cells/ml in a 24-well flat-bottom plate (TPP, Trasadingen, Switzerland) and stimulated for 24 h at 37°C and 5% $CO_2$ with PMA plus Io (250 ng/ml, Sigma). interleukin (IL)-2, interferon (IFN) γ, IL-4, IL-6, and IL-10 concentration from supernatants was quantified following the manufacturer's instructions for rat ELISA sets from BioSource (Nivelles, Belgium) and BD Pharmingen (Erembodegem, Belgium).

### 2.8. Splenocytes in vitro Ig production and serum Ig concentration

IgG and IgM produced by nonstimulated splenocytes for 7 days and serum IgG, IgM and IgA concentration were quantified using a sandwich ELISA technique as previously described [31]. Briefly, 96-well polystyrene plates (Nunc MaxiSorp, Wiesbaden, Germany) were coated with adequate dilution of capture antibodies (anti-rat IgG, IgM and IgA monoclonal antibodies). After samples and standards incubation, Ig isotypes were detected by adding biotin-conjugated anti-rat IgG, IgM or IgA antibodies followed by ExtrAvidin-peroxidase (Sigma). IgG, IgM and IgA standards and antibodies were provided by BD Pharmingen.

### 2.9. Statistical analysis

SPSS 16.0 (SPSS, Chicago, IL, USA) was used for the statistical analysis. Conventional one-way analysis of variance (ANOVA) was performed considering the experimental group as an independent variable. When CLA supplementation had a significant effect on the dependent variable, Bonferroni's and Scheffé's tests were applied. Significant differences were accepted at $P<.05$.

## 3. Results

### 3.1. Body weight

Rats' body weight was monitored daily throughout the study. Animals supplemented with CLA during suckling (4- and 3-week groups) exhibited higher body weight at the end of the study compared to that of animals only supplemented during early infancy (1-week group, $P<.05$) and to those that were non-supplemented (control group) (Table 1). Chow intake during the last week of study was similar among the four dietary groups (12–15 g/100 g rat). Moreover, no differences in BMI or Lee index were found among groups.

Table 1
Morphometrical parameters in 28-day-old rats[a]

| | 4 weeks | 3 weeks | 1 week | Control |
|---|---|---|---|---|
| Body weight (g) | 88.39±1.65*ϕ | 86.49±3.38ϕ | 70.21±3.24 | 75.74±3.75 |
| BMI (g/cm$^2$) | 0.41±0.01 | 0.39±0.01 | 0.37±0.01 | 0.37±0.01 |
| Lee index ($\sqrt[3]{}$g/cm) | 0.30±0.01 | 0.29±0.01 | 0.30±0.01 | 0.30±0.01 |

[a] Body weight, BMI and Lee index are expressed as mean±S.E.M. ($n=20$); 4-week group, pups supplemented with CLA during suckling by oral gavage and from 21 to 28 days old (early infancy) through solid diet; 3-week group, pups supplemented with CLA during suckling by oral gavage; 1 week group, pups supplemented with CLA diet only during early infancy. Control group, pups from reference group non-supplemented. Significant one way ANOVA differences: *$P<.05$ vs. control group; ϕ$P<.05$ vs. 1-week group.

### 3.2. Relative CLA concentration and fatty acid composition in rat plasma

*c*9,*t*11- and *t*10,*c*12-CLA isomers were quantified in plasma from 28-day-old animals (Table 2). Plasma from control rats had no *t*10,*c*12-CLA and a low content of *c*9,*t*11-CLA, which were lower than those found in the rest of the groups ($P<.05$). The animals who received CLA supplementation continuously during suckling and early infancy (4-week group) showed the highest content of both CLA isomers among the groups ($P<.05$). However, rats from the 3-week group (supplemented only during suckling and not during the last week) presented lower content of both CLA isomers in plasma than rats from the 1-week group (supplemented only after weaning) ($P<.05$). Moreover, the relative proportion between *c*9,*t*11- and *t*10,*c*12-CLA was higher in all CLA-supplemented groups (4-week, 91:9; 3-week, 90:10; 1-week, 94:6) than that of the original mixture (80:20).

Plasma fatty acid composition, as a percentage of total fatty acid content, is summarized in Table 3. Feeding CLA resulted in several changes in the fatty acid profile of plasma. The proportion of saturated, n-3 and n-6 fatty acids showed different patterns among groups. However, some common effects were observed due to CLA diet. The proportion of the major n-6 fatty acid, linoleic acid (C18:2), was significantly reduced in all CLA-supplemented groups ($P<.05$). Furthermore, the relative content of the longest chain n-3 PUFA (C22:5; C22:6) was significantly increased in animals receiving CLA for 1 and 3 week ($P<.05$). Although total n-3 and n-6 fatty acid content was not significantly different among groups, the 1-week group showed a higher plasma ratio of n-3/n-6 than the other groups ($P<.05$).

### 3.3. Serum immunoglobulin concentrations

Serum IgG, IgM and IgA concentrations were quantified in 28-day-old animals (Fig. 2). Rats from the control group had ~2 mg/ml of IgG

Table 2
Relative content of *c*9,*t*11- and *t*10,*c*12-CLA isomers as percentage of total fatty acids in plasma of 28-day-old pups[a]

| CLA isomer | 4 weeks | 3 weeks | 1 week | Control |
|---|---|---|---|---|
| *c*9,*t*11 | 1.31±0.03*ϕψ | 0.44±0.03*ϕ | 1.23±0.03*ψ | 0.15±0.01 |
| *t*10,*c*12 | 0.13±0.01*ϕψ | 0.05±0.00*ϕ | 0.08±0.00*ψ | N.D |

[a] All values are mean±S.E.M. (n = 10). 4-week group, pups supplemented with CLA during suckling by oral gavage and from 21- to 28-day-old (early infancy) through solid diet; 3-week group, pups supplemented with CLA during suckling by oral gavage; 1-week group, pups supplemented with CLA diet only during early infancy. Control group, non-supplemented pups from reference group. N.D, non-detectable. Significant one-way ANOVA differences: *$P<.001$ vs. control group; ϕ$P<.001$ vs. 1-week group; ψ$P<.001$ vs. 3-week group).

Table 3
Fatty acid composition of rat plasma of the four dietary groups (g/100 g of total fatty acids)[a]

| Group | | | | |
|---|---|---|---|---|
| Fatty Acid | 4 weeks | 3 weeks | 1 week | Control |
| C10:0 | 2.16±0.14$^{*\phi\psi}$ | 1.53±0.08$^{*\phi}$ | 1.41±0.07* | 1.99±0.08 |
| C12:0 | 1.09±0.10$^{*\phi}$ | 1.12±0.09$^{*\phi}$ | 0.78±0.04* | 1.02±0.06 |
| C14:0 | 2.08±0.07$^{*\phi\psi}$ | 1.52±0.08$^{*\phi}$ | 2.94±0.20* | 1.30±0.09 |
| C14:1 | 1.07±0.08$^{*\phi\psi}$ | 1.16±0.04$^{*\phi}$ | 0.71±0.03* | 0.98±0.10 |
| C16:0 | 19.65±0.34$^{\phi}$ | 19.75±0.50$^{\phi}$ | 23.21±0.44* | 19.55±0.78 |
| C16:1 | 1.10±0.08$^{*\phi\psi}$ | 0.90±0.05$^{*\phi}$ | 0.82±0.02* | 0.97±0.09 |
| C18:0 | 10.37±0.32$^{*\phi\psi}$ | 11.03±0.31$^{*\phi}$ | 9.78±0.21* | 10.63±0.29 |
| C18:1t n-9 | 0.00±0.00$^{\psi}$ | 0.04±0.01* | 0.04±0.00 | 0.00±0.00 |
| C18:1c n-9 | 11.35±0.24$^{\phi\psi}$ | 10.29±0.33$^{*\phi}$ | 10.77±0.47* | 11.50±0.26 |
| C18:1c n-7 | 1.43±0.04$^{*\phi\psi}$ | 1.11±0.06$^{*\phi}$ | 1.05±0.08* | 1.54±0.07 |
| C18:2t n-6 | 0.69±0.03$^{*\phi\psi}$ | 0.52±0.02$^{*\phi}$ | 0.27±0.02* | 0.43±0.02 |
| C18:2c n-6 | 26.52±0.55$^{*\phi\psi}$ | 27.55±0.84$^{*\phi}$ | 24.73±0.84* | 28.94±0.70 |
| C18:3 n-6 | 0.36±0.01$^{*\phi\psi}$ | 0.30±0.02* | 0.31±0.02* | 0.43±0.03 |
| C18:3 n-3 | 1.40±0.36 | 1.22±0.05* | 1.24±0.07* | 1.58±0.13 |
| C20:0 | 0.21±0.01$^{*\phi\psi}$ | 0.16±0.01$^{*\phi}$ | 0.10±0.01* | 0.17±0.01 |
| C18:2t | 1.44±0.04$^{*\phi\psi}$ | 0.49±0.03$^{*\phi}$ | 1.31±0.03* | 0.15±0.01 |
| C20:1 n-9 | 0.12±0.01$^{*\phi}$ | 0.12±0.01$^{*\phi}$ | 0.10±0.01* | 0.08±0.01 |
| C20:2 n-6 | 0.34±0.00$^{*\phi\psi}$ | 0.42±0.04$^{*\phi}$ | 0.25±0.03* | 0.30±0.02 |
| C20:3 n-6 | 0.42±0.02$^{\phi\psi}$ | 0.46±0.03$^{*\phi}$ | 0.58±0.05* | 0.42±0.02 |
| C20:4 n-6 | 13.69±0.19$^{\phi\psi}$ | 15.68±0.50$^{*\phi}$ | 14.53±0.48* | 13.65±0.37 |
| C22:0 | 0.18±0.01$^{*\phi\psi}$ | 0.05±0.01$^{*\phi}$ | 0.11±0.01* | 0.16±0.01 |
| C22:1 | 0.14±0.01$^{*\phi\psi}$ | 0.11±0.01$^{*\phi}$ | 0.04±0.00* | 0.12±0.01 |
| C22:2 | 0.34±0.02$^{*\phi\psi}$ | 0.31±0.02$^{*\phi}$ | 0.49±0.02* | 0.51±0.03 |
| C20:5 n-3 | 0.00±0.00* | 0.00±0.00* | 0.02±0.00* | 0.07±0.07 |
| C22:4 n-6 | 0.50±0.03$^{*\phi}$ | 0.51±0.03$^{*\phi}$ | 0.43±0.03 | 0.45±0.03 |
| C22:5 n-6 | 0.35±0.02$^{*\phi\psi}$ | 0.28±0.01 | 0.29±0.02 | 0.29±0.02 |
| C22:5 n-3 | 0.62±0.03$^{\phi\psi}$ | 0.77±0.02* | 0.79±0.04* | 0.63±0.02 |
| C22:6 n-3 | 2.28±0.03$^{\phi\psi}$ | 2.63±0.10$^{*\phi}$ | 2.84±0.10* | 2.27±0.11 |
| ∑ n-3 | 4.30±1.37 | 4.62±0.48 | 4.89±0.70 | 4.55±0.74 |
| ∑ n-6 | 42.87±11.60 | 45.72±19.31 | 41.39±22.43 | 44.91±18.75 |
| Ratio n-3/n-6 | 0.10±0.00$^{\phi}$ | 0.10±0.00$^{\phi}$ | 0.12±0.00* | 0.10±0.00 |

[a] Values are means±S.D. ($n$=10); 4-week group, pups supplemented with CLA during suckling by oral gavage and from 21 to 28 days old (early infancy) through solid diet; 3-week group, pups supplemented with CLA during suckling by oral gavage; 1-week group, pups supplemented with CLA diet only during early infancy. Control group, non-supplemented pups from reference group. Significant one-way ANOVA differences: $^{*}P$<.05 vs. control group; $^{\phi}P$<.05 vs. 1-week group; $^{\psi}P$<.05 vs. 3-week group).

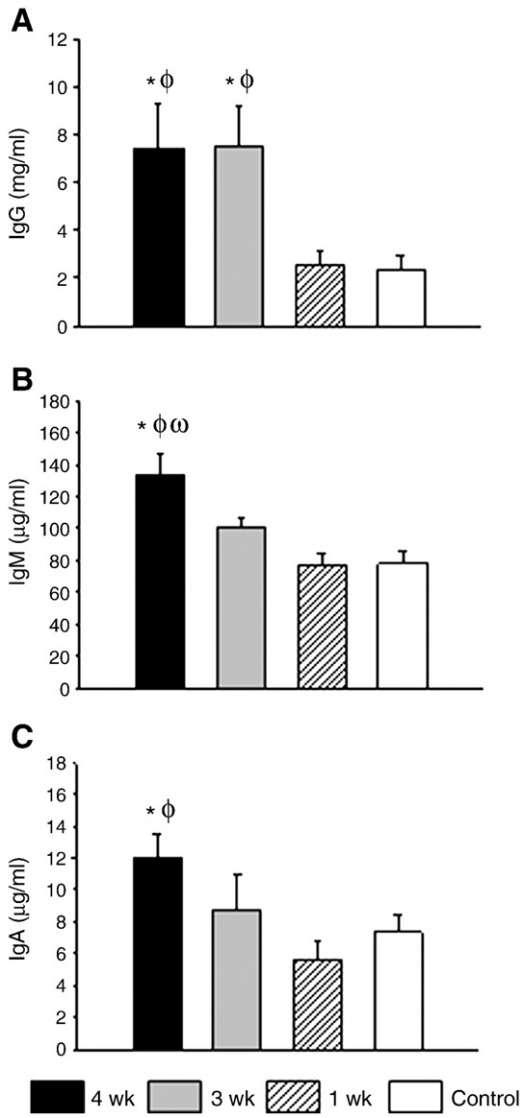


Fig. 2. Modulation of IgG (A), IgM (B) and IgA (C) sera concentration by dietary CLA. Values represent mean±S.E.M.; $n$=15–20 pups per group. Total period of supplementation was as follows: 4-week group, CLA supplementation during suckling by oral gavage and during early infancy through chow; 3-week group, suckling by oral gavage; 1-week group, CLA-fed pups during early infancy. Control group, non-supplemented animals. Significant one-way ANOVA differences: $^{*}P$<.05 vs. control group; $^{\phi}P$<.05 vs. 1-week group; $^{\omega}P$<.05 vs. 3-week group.

(Fig. 2A), ~80 µg/ml of IgM (Fig. 2B) and ~8 µg/ml of IgA (Fig. 2C). Animals that received CLA diet during and after suckling (4-week group) showed higher IgG, IgM and IgA sera concentrations than those receiving CLA only for 1 week after suckling and rats from the control group ($P$<.05). Moreover, rats from the 3-week group, i.e., those receiving CLA diet only during suckling, showed 3 times the IgG concentration than those from the 1 week and control groups ($P$<.05). Considering total serum Ig (data not shown), CLA supplementation during suckling (4- and 3-week groups) increased the Ig concentration fourfold compared to the 1-week and control groups, mainly by increasing the predominant in vivo Ig isotype concentration, IgG.

### 3.4. Spleen lymphocyte proliferation, viability and cytokine secretion

CLA supplementation during suckling (4- and 3-week groups) reduced ex vivo spleen lymphoproliferative ability compared to that in the 1-week and Control groups, but it was only significant for the 4-week group ($P$<.05) (Fig. 3A). CLA supplementation also confers more resistance to the mitogen toxic effects, the 4-week group showing the lowest mortality among the groups ($P$<.05) (Fig. 3B). Thus, the decrease in the proliferation rate cannot be attributed to a lower viability caused by CLA diet. On the other hand, after stimulating splenocytes for 24 h, IL-2, IFNγ, IL-4 and IL-10 concentration in the culture medium was not significantly modified in any CLA dietary group (Table 4). However, IL-6 concentration was increased in the 4-week group compared to the 3-week and 1-week groups ($P$<.05), while IL-6 was not detected in the control group (Table 4).

### 3.5. In vitro spleen immunoglobulin production

After 7 days of splenocyte culture, spontaneous production of IgM and IgG was quantified. Under these conditions, IgM was the predominant isotype found in supernatants, being ~100 times higher than IgG production ($P$<.05) (Fig. 4). The groups supplemented with CLA during suckling (4- and 3-week groups) synthesised higher IgM and IgG levels than the control group, but it was only significant in the 3-week group ($P$<.05). Spleen Ig production from the 1-week and control groups was similar.

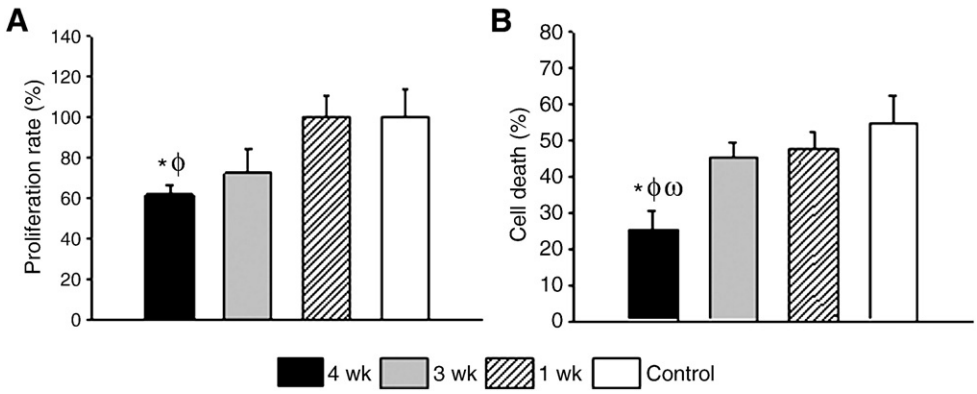


Fig. 3. CLA effects on proliferation rate (A) and cell death (B) in mitogen-stimulated spleen lymphocytes. Values represent mean±S.E.M.; $n$=15–20 pups per group. Total period of supplementation was as follows: 4-week group, CLA supplementation during suckling by oral gavage and during early infancy through chow; 3-week group, suckling by oral gavage; 1-week group, CLA-fed pups during early infancy. Control group, nonsupplemented animals. Significant one-way ANOVA differences: $^{*}P<.05$ vs. control; $^{\phi}P<.05$ vs. 1-week group; $^{\omega}P<.05$ vs. 3-week group.

## 4. Discussion

The neonatal immune functionality of mammals is very limited, and during early life, it gradually starts to acquire the immune status of adults [32,33]. The impact of nutritional fatty acids on the development of the early immune system has been demonstrated to involve regulation of metabolic processes and gene expression of important proteins such as enzymes and cytokines [34]. Some studies have evaluated the effect of CLA isomer mixtures on immune function in adult animals at doses ranging from 0.1% to 2% of diet (w/w) [35]. Moreover, in previous studies, we have shown the effects of CLA on the immune system in neonatal rats [21]. In the present study, we establish the immunomodulatory effects of an 80:20 mixture of *c*9, *t*11- and *t*10,*c*12-CLA after weaning on rats supplemented with CLA from birth until the end of suckling and/or early infancy.

In the present study we observed that CLA was absorbed by all the supplemented animals since *c*9,*t*11- and *t*10,*c*12-CLA isomers were detected in the plasma of all 28-day-old rats fed CLA. These results match previous data obtained in 21-day-old animals [21] and with other PUFAs studies using similar experimental designs [22,36]. The highest content of CLA in plasma was achieved in the longest and continuous CLA supplementation (4-week group), whereas the group that received CLA for 3 week and then nothing for 1 week exhibited lower CLA plasma content than the 1-week group which only received CLA for the last week. Previous interventional studies have shown that the proportion of fatty acids in the dietary supplement is highly correlated to that one found in plasma and membrane [37,38]. Therefore, we can suggest that in our study, just finishing the supplementation period, CLA plasma levels are an indicative measure of the CLA incorporated in the cell membranes, in our case for 4 week and 1 week group. But this is not the case for the 3-week group due to the time lapse between the last CLA intake and the day of quantification of CLA plasma content. The CLA membrane incorporation suggested here agrees with the study of Subbaiah et al. [39], which demonstrates that CLA is incorporated into cell membranes to varying extent, depending upon the experimental conditions.

Table 4
Content of cytokines in 24-h splenocytes supernatants after mitogen stimulation[a]

| Cytokines | 4 weeks | 3 weeks | 1 week | Control |
|---|---|---|---|---|
| Th1 | | | | |
| IL-2 (ng/ml) | 6.2±0.8 | 6.1±0.8 | 7.2±1.1 | 6.8±1.2 |
| IFNγ (ng/ml) | 10.9±1.4 | 7.1±0.9 | 7.3±0.8 | 6.0±1.3 |
| Th2 | | | | |
| IL-4 (pg/ml) | 27.8±4.0 | 18.8±2.8 | 28.7±7.4 | 17.3±2.4 |
| IL-10 (pg/ml) | 244.4±41.0 | 224.9±29.2 | 222.4±34.5 | 191.6±24.2 |
| IL-6 (pg/ml) | 219.0±38.0*ϕ | 40.1±12.9 | 45.7±15.1 | N.D. |

[a] Data are expressed as mean±S.E.M. ($n$=10); 4-week group, pups supplemented with CLA during suckling and early infancy; 3-week group, pups supplemented with CLA during suckling; 1-week group, pups supplemented with CLA during early infancy. Control group, pups nonsupplemented. N.D., non-detectable. Significant one-way ANOVA differences: $^{*}P<.05$ vs. 3-week; $^{\phi}P<.05$ vs. 1-week group.

On the other hand, the relative proportion of *c*9,*t*11/*t*10,*c*12 in the plasma of CLA-supplemented animals (~90:10) is higher than in the original isomer mixture (80:20) administered to the animals. These changes in proportions could be attributed to the fact that *t*10,*c*12-CLA isomer is more easily oxidized because this isomer activates the β-oxidation system more strongly than *c*9,*t*11-CLA [23,40]. Moreover, although it has been described that both isomers present similar absorption rates in adult rat intestine [23], it is possible that young rodents preferentially absorb the *c*9,*t*11 isomer. Additionally, although no *t*10,*c*12-CLA was found in the plasma of control animals, small quantities of *c*9,*t*11-CLA isomer were detected, which would indicate an endogenous production of *c*9,*t*11-CLA, as previously described [41], and, therefore, its influence on the *c*9,*t*11/*t*10,*c*12 ratio observed in plasma. In any case, the absorption of CLA modified the rat plasma fatty acid profile differentially among the groups, but without changing total n-3 and n-6 fatty acids. However, it is important to note that the n-3/n-6 ratio was higher in the 1-week group than in the others, mainly due to the strong reduction of linoleic acid. Overall, CLA supplementation reduced the major n-6 fatty acid in all supplemented groups and increased the main n-3 fatty acids, such as DPA and DHA, in the 3- and 1-week groups. These changes found in the plasma of weaned rats after CLA feeding had no defined pattern and are in accordance with variations found in abdominal and muscle lipids of CLA-fed adult rats [42]. Therefore, we can suggest that changes in immune response found here after CLA supplementation are mainly due to CLA rather than by modifications in fatty acid profile.

CLA supplementation from birth during suckling and/or early infancy increased body weight at the age of 28 days. However, in spite of increasing body weight, CLA supplementation did not modify morphometrical parameters such as BMI or Lee index, which are similar to those reported for non-supplemented rats at a similar age [43], indicating overall a CLA growth improvement. Since the *c*9,*t*11-CLA isomer represents 80% of the mixture used, our results are in line with the CLA growth effect obtained by the *c*9,*t*11-CLA isomer shown by Pariza et al. [12].

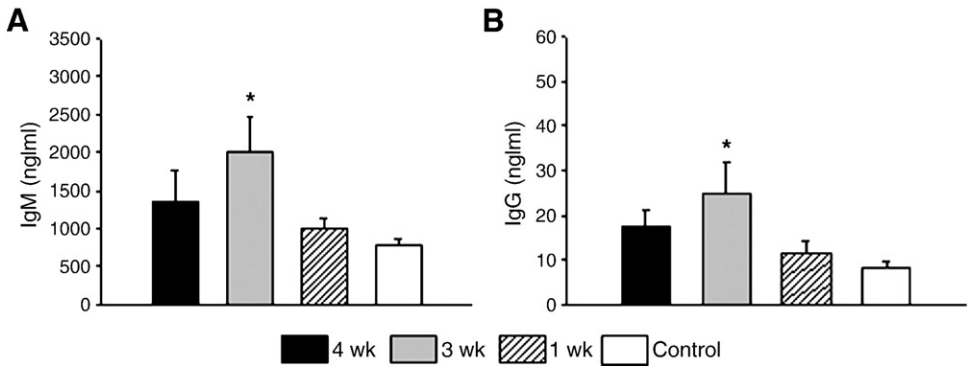


Fig. 4. Dietary CLA changes in splenocyte Ig production. IgM (A) and IgG (B) concentrations in supernatants after 7 days of spleen cell culture. Values represent mean±S.E.M.; $n$=15–20 pups per group. Total period of supplementation was as follows: 4-week group, CLA supplementation during suckling by oral gavage and during early infancy through chow; 3-week group, suckling by oral gavage; 1-week group, CLA-fed pups during early infancy. Control group, non-supplemented animals. Significant one-way ANOVA differences: $^{*}P<.05$ vs. control group.

Regarding CLA immune effects, the supplementation of *c*9,*t*11- and *t*10,*c*12-CLA isomers at 80:20 ratio enhances antibody synthesis in infant rats, specifically IgG, IgM and IgA isotypes. This effect is clearly evidenced in animals receiving dietary CLA continuously during and after suckling. Moreover, this immunoenhancing effect correlates with the IgA increase found in the intestinal compartment shown in previous studies, where both IgA gene expression and protein were up-regulated by CLA [44]. The effect of CLA on in vivo antibody synthesis partially agrees with results obtained from isolated splenocytes. Both in vivo and ex vivo CLA-immunopotentiating effects are higher in 28-day-old animals than those described for neonatal [21] and, moreover, for adult animals where no effect is found [20]. Overall, this particular CLA effect seems to be specific to early life (suckling and infancy), in which antibody production is still in development in order to control the arrival of new antigens from ingestion of breast milk during suckling or solid diet thereafter.

Moreover, the animals supplemented only during suckling (3-week group) developed high IgG serum concentration and high ex vivo splenocyte IgM and IgG production, thus indicating the lasting of the CLA effect on the immunoglobulin synthesis later in life, or at least 1 week after finishing the CLA intake.

Studies in isolated splenocytes from animals fed CLA allow an approach to the mechanism involved in the immunomodulatory effect of this fatty acid. Mitogen-induced splenocyte proliferation was down-modulated in the longer-lasting CLA supplementation, similarly to that described in adults [18–20]. However, this effect was not observed in 21-day-old animals, probably due to the immaturity of this function (which is acquired later in life) at a very early age [31]. Thus, the age of 28 days is optimal to evaluate CLA effects, since at this age, CLA still increases antibody production and is also able to modulate lymphoproliferation. Calder and Newsholme [45] reported that some PUFAs inhibited lymphocyte proliferation without decreasing IL-2 concentration, a fact that is consistent with the present results showing that CLA supplementation did not modify IL-2 production. In this sense, many studies have described conformation changes in IL-2 receptors by PUFAs, specifically modifying lipid rafts [46,47]. Thus, CLA, even with *trans* double bonds, could potentially alter membrane structure, including lipid rafts, by preventing IL-2Rα migration to soluble membranes, where IL-2 signalling occurs and T-cell activation and proliferation are consequently induced [46,48,49]. In addition, the antiproliferative lymphocyte effect exhibited by dietary CLA in the present study could be mediated by the nuclear peroxisome proliferator-activated receptor (PPAR)γ, because intestinal PPARγ gene expression has been found up-regulated after CLA supplementation [44]. The PPARγ-dependent CLA effect was first described by Bassaganya-Riera and Hontecillas [50] in a pig-inflammatory bowel disease (IBD) model, where dietary CLA resulted in IBD amelioration and PPARγ gene expression up-regulation.

Besides proliferative response, the cytokine production in isolated splenocytes was also determined. The CLA diet did not affect the in vitro production of IFNγ and IL-2 (Th1 cytokines). Regarding Th2 cytokines, proteins involved in T-cell polarization to antibody production, IL-4 and IL-10 concentration were similar in all groups. However, interestingly, IL-6 production was higher in all the CLA-diet groups and especially in the longest diet group (4-week group) which produced the strongest enhancing effect on antibody synthesis. These results are not in line with those obtained in adult humans described by Albers et al. [17] and Sofi et al. [51]. Since IL-6 is clearly defined as a prominent regulator of T-cell proliferation and differentiation of Ig-secreting B cells [52], it might be suggested that the effects on proliferation and antibody production induced by CLA could be due to an up-regulation of IL-6 production. Further studies should confirm this hypothesis and ascertain the mechanism involved.

In summary, although further studies should be carried out to elucidate CLA signalling mechanisms, these data support the early life immune-enhancing properties of *c*9,*t*11-CLA, the main CLA isomer present in breast milk. The results presented herein demonstrate that supplementation with an 80:20 *cis*-9,*trans*-11:*trans*-10,*cis*-12-CLA mix during suckling and early infancy enhances antibody synthesis and down-regulates lymphoproliferative response as described for neonatal and adult rats, respectively.

## Acknowledgments

The present study was supported by partial funding from the Generalitat de Catalunya (SGCR-2005-00833). CC and AF acknowledge partial funding for this research from the CIBER Epidemiología y Salud Pública (CIBERESP), Spain. The oil used in the study was a gift from Loders Croklaan, Lipid Nutrition, Wormerveer, The Netherlands.

## References

[1] Field CJ, Van Aerde JE, Robinson LE, Clandinin MT. Effect of providing a formula supplemented with long-chain polyunsaturated fatty acids on immunity in full-term neonates. Br J Nutr 2008;99:91–9.
[2] Kelly D, Coutts AG. Early nutrition and the development of immune function in the neonate. Proc Nutr Soc 2000;59:177–85.
[3] Gil A, Rueda R. Interaction of early diet and the development of the immune system. Nutr Res Rev 2002;15:263–92.
[4] Field CJ, Clandinin MT, Van Aerde JE. Polyunsaturated fatty acids and T-cell function: implications for the neonate. Lipids 2001;36:1025–32.

[5] Pérez-Cano FJ, Marín-Gallén S, Castell M, Rodríguez-Palmero M, Rivero M, Franch À, et al. Bovine whey protein concentrate supplementation modulates maturation of immune system in suckling rats. Br J Nutr 2007;98:S80–84.
[6] Hanson LA, Korotkova M, Lundin S, Haversen L, Silfverdal SA, Mattsby Baltzer I, et al. The transfer of immunity from mother to child. Ann N Y Acad Sci 2003;987: 199–206.
[7] Pariza MW, Ashoor SH, Chu FS. Mutagens in heat-processed meat, bakery and cereal products. Food Cosmet Toxicol 1979;17:429–30.
[8] McGuire MK, Park Y, Behre RA, Harrison LY, Shultz TD, McGuire MA. Conjugated linoleic acid concentration of human milk and infant formula. Nutr Res 1997;17: 1277–83.
[9] Nicolosi RJ, Rogers EJ, Kritchevsky D, Scimeca JA, Huth PJ. Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. Artery 1997;22:266–77.
[10] Lee JH, Cho KH, Lee KT, Kim MR. Antiatherogenic effects of structured lipid containing conjugated linoleic acid in C57BL/6J mice. J Agric Food Chem 2005;53: 7295–301.
[11] de Deckere EA, van Amelsvoort JM, McNeill GP, Jones P. Effects of conjugated linoleic acid (CLA) isomers on lipid levels and peroxisome proliferation in the hamster. Br J Nutr 1999;82:309–17.
[12] Pariza MW, Park Y, Cook ME. The biologically active isomers of conjugated linoleic acid. Prog Lipid Res 2001;40:283–98.
[13] Terpstra AH. Effect of conjugated linoleic acid on body composition and plasma lipids in humans: an overview of the literature. Am J Clin Nutr 2004; 79:352–61.
[14] Chin SF, Storkson JM, Albright KJ, Cook ME, Pariza MW. Conjugated linoleic acid is a growth factor for rats as shown by enhanced weight gain and improved feed efficiency. J Nutr 1994;124:2344–9.
[15] Ma DW, Wierzbicki AA, Field CJ, Clandinin MT. Conjugated linoleic acid in canadian dairy and beef products. J Agric Food Chem 1999;47:1956–60.
[16] Navarro V, Fernández-Quintela A, Churruca I, Portillo MP. The body fat-lowering effect of conjugated linoleic acid: a comparison between animal and human studies. J Physiol Biochem 2006;62:137–47.
[17] Albers R, van der Wielen RPJ, Brink EJ, Hendriks HFJ, Dorovska-Taran VN, Mohede ICM. Effects of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 conjugated linoleic acid (CLA) isomers on immune function in healthy men. Eur J Clin Nutr 2003;57: 595–603.
[18] Bassaganya-Riera J, Hontecillas R, Zimmerman DR, Wannemuehler MJ. Long-term influence of lipid nutrition on the induction of CD8(+) responses to viral or bacterial antigens. Vaccine 2002;20:1435–44.
[19] Bassaganya-Riera J, Pogranichniy RM, Jobgen SC, Halbur PG, Yoon KJ, O'Shea M, et al. Conjugated linoleic acid ameliorates viral infectivity in a pig model of virally induced immunosuppression. J Nutr 2003;133:3204–14.
[20] Ramírez-Santana C, Castellote C, Castell M, Rivero M, Rodríguez-Palmero M, Franch À, et al. Long-term feeding of the *cis*-9,*trans*-11 isomer of conjugated linoleic acid reinforces the specific immune response in rats. J Nutr 2009;139: 76–81.
[21] Ramírez-Santana C, Pérez-Cano FJ, Castellote C, Castell M, Rivero M, Rodríguez-Palmero M, et al. Higher immunoglobulin production in conjugated linoleic acid-supplemented rats during gestation and suckling. Br J Nutr 2009;102: 858–68.
[22] Porsgaard T, Xu X, Mu H. The form of dietary conjugated linoleic acid does not influence plasma and liver triacylglycerol concentrations in Syrian golden hamsters. J Nutr 2006;136:2201–6.
[23] Tsuzuki T, Ikeda I. Slow absorption of conjugated linoleic acid in rat intestines, and similar absorption rates of 9c,11t-conjugated linoleic acid and 10t,12c-conjugated linoleic acid. Biosci Biotechnol Biochem 2007;71:2034–40.
[24] Yamasaki M, Kishihara K, Mansho K, Ogino Y, Kasai M, Sugano M, et al. Dietary conjugated linoleic acid increases immunoglobulin productivity of Sprague-Dawley rat spleen lymphocytes. Biosci Biotechnol Biochem 2000;64: 2159–64.
[25] Yamasaki M, Chujo H, Hirao A, Koyanagi N, Okamoto T, Tojo N, et al. Immunoglobulin and cytokine production from spleen lymphocytes is modulated in C57BL/6J mice by dietary *cis*-9, *trans*-11 and *trans*-10, *cis*-12 conjugated linoleic acid. J Nutr 2003;133:784–8.
[26] Bassaganya-Riera J, Reynolds K, Martino-Catt S, Cui Y, Hennighausen L, Gonzalez F, et al. Activation of PPAR gamma and delta by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. Gastroenterology 2004;127:777–91.
[27] Frazier AL, Ryan CT, Rockett H, Willett WC, Colditz GA. Adolescent diet and risk of breast cancer. Breast Cancer Res 2003;5:R59–R64.
[28] Reeves PG, Nielsen FH, Fahey GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;123:1939–51.
[29] Bondía-Pons I, Moltò-Puigmartí C, Castellote AI, López-Sabater MC. Determination of conjugated linoleic acid in human plasma by fast gas chromatography. J Chromatogr A 2007;1157:422–9.
[30] Pérez-Cano FJ, Castellote C, Marín-Gallén S, Franch À, Castell M. Neonatal immunoglobulin secretion and lymphocyte phenotype in rat small intestine lamina propria. Pediatr Res 2005;58:164–9.
[31] Pérez-Cano FJ, Castellote C, Marín-Gallén S, González-Castro A, Franch À, Castell M. Phenotypic and functional characteristics of rat spleen lymphocytes during suckling. Dev Comp Immunol 2007;31:1264–77.
[32] Adkins B. T-cell function in newborn mice and humans. Immunol Today 1999;20: 330–5.
[33] Piguet PF, Irle C, Kollatte E, Vassalli P. Post-thymic T lymphocyte maturation during ontogenesis. J Exp Med 1981;154:581–93.
[34] Enke U, Seyfarth L, Schleussner E, Markert UR. Impact of PUFA on early immune and fetal development. Br J Nutr 2008;30:1–11.
[35] Kelley DS, Erickson KL. Modulation of body composition and immune cell functions by conjugated linoleic acid in humans and animal models: benefits vs. risks. Lipids 2003;38:377–86.
[36] Morgado N, Rigotti A, Valenzuela A. Comparative effect of fish oil feeding and other dietary fatty acids on plasma lipoproteins, biliary lipids, and hepatic expression of proteins involved in reverse cholesterol transport in the rat. Ann Nutr Metab 2005;49:397–406.
[37] Marín M, Rey G, Rodrigo M, Alaniz M. Ácidos grasos de fosfolípidos en plasma y eritrocitos de lactantes desnutridos alimentados con leche materna o formulas. Medicina (Buenos Aires) 2001;61:41–8.
[38] Cao J, Schwichtenberg KA, Hanson NQ, Tsai MY. Incorporation and clearance of omega 3 fatty acids in erythrocyte membranes and plasma phospholipids. Clin Chem 2006;52:2265–72.
[39] Subbaiah P, Sircar D, Aizezi B, Mintzer E. Differential effects of conjugated linoleic acid isomers on the biophysical and biochemical properties of model membranes. Biochim Biophys Acta 1798;2010:506–14.
[40] Bowen RA, Clandinin MT. Maternal dietary 22:6n-3 is more effective than 18:3n-3 in increasing the 22:6n-3 content in phospholipids of glial cells from neonatal rat brain. Br J Nutr 2005;93:601–11.
[41] Chin SF, Storkson JM, Liu W, Albright KJ, Pariza MW. Conjugated linoleic acid (9,11- and 10,12-octadecadienoic acid) is produced in conventional but not germ-free rats fed linoleic acid. J Nutr 1994;124:694–701.
[42] Szymczyk B, Pisulewski P, Szczurek W, Hanczakowski P. The effects of feeding conjugated linoleic acid (CLA) on rat growth performance, serum lipoproteins and subsequent lipid composition of selected rat tissues. J Sci Food Agric 2000;80: 1553–8.
[43] Novelli EL, Diniz YS, Galhardi CM, Ebaid GM, Rodrígues HG, Mani F, et al. Anthropometrical parameters and markers of obesity in rats. Lab Anim 2007;41: 111–9.
[44] Pérez-Cano FJ, Ramírez-Santana C, Molero-Luís M, Castell M, Rivero M, Castellote C, et al. Mucosal IgA increase in rats by continuous CLA feeding during suckling and early infancy. J Lipid Res 2009;50:467–76.
[45] Calder PC, Newsholme EA. Polyunsaturated fatty acids suppress human peripheral blood lymphocyte proliferation and interleukin-2 production. Clin Sci (Lond) 1992;82:695–700.
[46] Yaqoob P, Newsholme EA, Calder PC. The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation. Immunology 1994;82:603–10.
[47] Schley PD, Brindley DN, Field CJ. (n-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. J Nutr 2007;137:548–53.
[48] Marmor MD, Julius M. Role for lipid rafts in regulating interleukin-2 receptor signaling. Blood 2001;98:1489–97.
[49] Ferreri C, Panagiotaki M, Chatgilialoglu C. Trans fatty acids in membranes: the free radical path. Mol Biotechnol 2007;37:19–25.
[50] Bassaganya-Riera J, Hontecillas R. CLA and n-3 PUFA differentially modulate clinical activity and colonic PPAR-responsive gene expression in a pig model of experimental IBD. Clin Nutr 2006;25:454–65.
[51] Sofi F, Buccioni A, Cesari F, Gori AM, Minieri S, Mannini L, et al. Effects of a dairy product (pecorino cheese) naturally rich in cis-9, trans-11 conjugated linoleic acid on lipid, inflammatory and haemorheological variables: a dietary intervention study. Nutr Metab Cardiovasc 2010;20:117–24.
[52] Jones SA. Directing transition from innate to acquired immunity: defining a role for IL-6. J Immunol 2005;175:3463–8.