

## Dietary *trans*-10, *cis*-12-conjugated linoleic acid alters fatty acid metabolism and microbiota composition in mice

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### Abstract

The main aim of the present study was to investigate the effects of dietary *trans*-10, *cis*-12-conjugated linoleic acid (*t10c12*-CLA) on intestinal microbiota composition and SCFA production. C57BL/6 mice (*n* 8 per group) were fed a standard diet either supplemented with *t10c12*-CLA (0.5%, w/w) (intervention) or with no supplementation (control), daily for 8 weeks. Metabolic markers (serum glucose, leptin, insulin and TAG, and liver TAG) were assessed by ELISA commercial kits, tissue long-chain fatty acids and caecal SCFA by GC, and microbial composition by 16S rRNA pyrosequencing. Dietary *t10c12*-CLA significantly decreased visceral fat mass ( $P < 0.001$ ), but did not affect body weight (intervention), when compared with no supplementation (control). Additionally, lipid mass and composition were affected by *t10c12*-CLA intake. Caecal acetate, propionate and isobutyrate concentrations were higher ( $P < 0.05$ ) in the *t10c12*-CLA-supplemented group than in the control group. The analysis of the microbiota composition following 8 weeks of *t10c12*-CLA supplementation revealed lower proportions of Firmicutes ( $P = 0.003$ ) and higher proportions of Bacteroidetes ( $P = 0.027$ ) compared with no supplementation. Furthermore, *t10c12*-CLA supplementation for 8 weeks significantly altered the gut microbiota composition, harbouring higher proportions of Bacteroidetes, including Porphyromonadaceae bacteria previously linked with negative effects on lipid metabolism and induction of hepatic steatosis. These results indicate that the mechanism of dietary *t10c12*-CLA on lipid metabolism in mice may be, at least, partially mediated by alterations in gut microbiota composition and functionality.

**Key words:** *trans*-10, *cis*-12-Conjugated linoleic acid: Intestinal microbiota: Lipid metabolism

Conjugated linoleic acid (CLA) is a class of isomers of linoleic acid that occur naturally in dairy products and meat from ruminant animals due to bacterial biohydrogenation of ingested PUFA in the rumen<sup>(1)</sup>. CLA has been shown to be produced *in vitro* and *in vivo* by different species of bacteria<sup>(2–5)</sup>. Although *cis*-9, *trans*-11-CLA (rumenic acid) is the major natural form/isomer of CLA in foods, accounting for more than 90% of dietary CLA, mixtures containing equal amounts of *cis*-9, *trans*-11-CLA and *trans*-10, *cis*-12-CLA (*t10c12*-CLA) isomers are produced industrially and sold as supplements. Both the isomers exhibit significant biological activities, which may exert synergistic or antagonistic effects<sup>(1)</sup>. CLA has been shown to inhibit carcinogenesis<sup>(6)</sup>, prevent atherosclerosis in different animal models<sup>(7–9)</sup>, modulate the immune system<sup>(10,11)</sup>, and affect

body composition, by reducing body fat and increasing lean body mass<sup>(12,13)</sup>. Also, *t10c12*-CLA has been shown to be the isomer responsible for the anti-obesity effect attributed to CLA, and over the past years, its impact on body fat modulation has been largely studied in different animal models and in human subjects<sup>(12–14)</sup>. There is evidence to suggest that fat mass reduction is the result of multiple interactions of *t10c12*-CLA with numerous metabolic signalling pathways, leading to decreased energy intake and increased energy expenditure, inhibition of adipogenesis and lipogenesis, modulation of adipokines and cytokines, and increased fatty acid (FA)  $\beta$ -oxidation in skeletal muscle<sup>(15)</sup>.

The response to CLA supplementation appears to be highly species-specific, with mice generally being more sensitive

**Abbreviations:** *t10c12*-CLA, *trans*-10, *cis*-12-conjugated linoleic acid; FA, fatty acids; SREBP-1c, sterol regulatory element-binding protein 1c.

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than other animal models and human subjects. These differences are attributed to the dose levels used (human trials use lower doses), age (animal trials usually use young subjects), rate of body fat turnover (small animals have faster metabolism) and dietary regimens (*ad libitum* in animal models *v.* energy restriction in human trials)<sup>(15,16)</sup>. In most studies using mice, body fat reduction induced by  $\text{\textit{t10c12-CLA}}$  supplementation is accompanied by adverse effects such as hepatic steatosis and hyperinsulinaemia<sup>(12,13)</sup>. These are features frequently associated with the metabolic syndrome, and commonly observed in obese and diabetic individuals<sup>(17,18)</sup>. Hepatic steatosis is characterised by an increase in liver mass with the accumulation of intracellular lipids, primarily in the form of TAG. Increased uptake of circulating FA, increased hepatic *de novo* lipogenesis, reduced rate of FA oxidation and reduced FA secretion are the multiple mechanisms leading to increased accumulation of lipids in the liver<sup>(19)</sup>.

Moreover, recent studies have identified the gut microbiota as an environmental factor with an important role in host fat metabolism, and in the development of hepatic steatosis<sup>(17)</sup>. In a continuous bidirectional communication between the gut and the liver, hepatic products can directly influence the microbiota composition, whereas bacterial metabolites may have both direct and indirect effects on liver function and physiology<sup>(20,21)</sup>. Thus, in the present study, we investigated the impact of dietary  $\text{\textit{t10c12-CLA}}$  supplementation on intestinal microbiota composition and SCFA production.

## Materials and methods

### Animals and diets

Experiments involving animals were carried out in accordance with the protocols approved by the Ethics Committee, University College Cork, under a license issued by the Department of Health and Children (Cruelty to Animal Act 1876, Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (89/609/EEC)). Male C57BL/6 mice (7–8 weeks of age) were obtained from Charles River, and housed under barrier-maintained conditions within the Biological Services Unit, University College Cork. After 1 week of acclimatisation, the animals were divided into two groups (*n* 8 per group). Both groups were fed *ad libitum* with a Teklad Global rodent standard diet (#2018S; Harlan Laboratories) and allowed free access to water. The FA present in the diet included palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid. The intervention group received the standard diet supplemented with 0.5% (w/w)  $\text{\textit{t10c12-CLA}}$  (Matreya LLC; Table 1). Body weight was assessed weekly. After 8 weeks of dietary intervention, fasted animals were killed by decapitation, and blood samples were collected, allowed to clot for 2 h at 4°C and centrifuged at 2000 **g** for 20 min. Liver, brain, fat pads (epididymal, perirenal and mesenteric), heart, kidneys and intestines were removed, blotted dry on a filter paper, weighed and flash-frozen in liquid N<sub>2</sub>. Caecal content was collected for the analysis of microbiota composition and SCFA concentrations. All samples were stored at –80°C before analyses.

**Table 1.** Diet composition (g/100 g)

Ingredients	$\text{\textit{t10c12-CLA}}$ -supplemented	Unsupplemented
Protein	18.6	18.6
Carbohydrate	44.2	44.2
Fat	6.7	6.2
Fatty acids		
Palmitic	0.7	0.7
Stearic	0.2	0.2
Oleic	1.2	1.2
Linoleic	3.1	3.1
Linolenic	0.3	0.3
$\text{\textit{t10c12-CLA}}$	0.5	–
Amino acids	18.4	18.4
Vitamins and minerals	3.7	3.7

$\text{\textit{t10c12-CLA}}$ , *trans*-10, *cis*-12-conjugated linoleic acid.

### Lipid extraction and fatty acid analysis

FA profiles of the liver, brain, epididymal adipose tissue, heart and kidneys were analysed. Lipids were extracted with chloroform–methanol (Fisher Scientific), according to the method of Folch *et al.*<sup>(22)</sup>. After extraction, samples were methylated by using 0.5 M-NaOH (Sigma-Aldrich) in methanol for 10 min at 90°C, followed by 10 min incubation at 90°C with 14% (w/v) boron trifluoride in methanol (Sigma-Aldrich)<sup>(23)</sup>. FA methyl esters were recovered with hexane (Fisher Scientific). Before GLC analysis, samples were dried over anhydrous sodium sulphate (Sigma-Aldrich) for 1 h, and stored at –20°C. FA methyl esters were separated using a Varian 3800 GC flame-ionisation system, fitted with a Chrompack CP Sil 88 column (Chrompack; 100 m × 0.25 mm internal diameter, 0.20 μm film thickness), and He gas as a carrier. The column oven was programmed initially at 80°C for 8 min, and then increased by 8.5°C/min to a final column temperature of 200°C. The injection volume used was 0.6 μl, with automatic sample injection on an SPI 1093 splitless on-column temperature-programmable injector. Peaks were integrated using the Varian Star Chromatography Workstation version 6.0 software, and identified by comparison of retention times with pure FA methyl ester standards (Nu-Chek Prep, Inc.). The percentage of individual FA was calculated according to the peak areas, relative to the total area (total FA were set at 100%). All FA results are expressed as g/100 g FA methyl ester.

### SCFA concentration analysis

Caecal content was vortex-mixed with Milli-Q water, incubated at room temperature for 10 min, and centrifuged at 10 000 **g** to pellet bacteria and other solids. The supernatant was filtered and transferred to a clear GC vial. 2-Ethylbutyric acid (Sigma) was used as the internal standard. The concentration of SCFA was analysed using a Varian 3500 GC flame-ionisation system, fitted with a Nukol-FFAP column (30 m × 0.32 mm × 0.25 μm; Sigma). The initial oven temperature was set at 100°C for 0.5 min, raised to 180°C at 8°C/min and held for 1 min, then increased to 200°C at 20°C/min, and finally held at 200°C for 5 min. The temperatures of the injector and the detector were set at 240 and 250°C, respectively.

He gas was used as a carrier at a flow rate of 1.3 ml/min. A standard curve was built with different concentrations of a standard mix containing acetate, propionate, isobutyrate and *n*-butyrate (Sigma). Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software. All SCFA data are expressed as  $\mu\text{mol/g}$ .

### Measurement of hepatic TAG concentrations

Hepatic lipids were extracted according to the method of Folch *et al.*<sup>(22)</sup>. After extraction, samples were dried under a stream of  $\text{N}_2$ , and resuspended in a 5% (v/v) solution of Triton X-100 in distilled water. TAG concentrations were measured using a commercial kit (EnzyChrom Triglyceride Assay; BioAssay Systems).

### Measurement of serum metabolic markers

Commercial kits were used for the measurement of metabolic markers in serum. Glucose concentration was measured using the QuantiChrom Glucose Assay Kit (BioAssay Systems), TAG concentration using the EnzyChrom Triglyceride Assay Kit (BioAssay Systems), insulin concentration using the Ultra-Sensitive Mouse ELISA Kit (Crystal Chem, Inc.), and leptin concentration using the Mouse Leptin ELISA Kit (Crystal Chem, Inc.).

### Amplicon sequencing of the gut microbiota

For high-throughput amplicon sequencing, DNA was extracted from faecal samples using the QIAmp DNA Stool Mini Kit (Qiagen), according to the manufacturer's instructions, with the addition of a bead-beating step (30 s,  $\times 3$ ), and stored at  $-20^\circ\text{C}$ . The microbiota composition of the samples was established by amplicon sequencing of the 16S rRNA gene V4; universal 16S rRNA primers estimated to bind to 94.6% of all 16S genes (i.e. the forward primer (F1: 5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers (R1: 5'-TACCRGGGTHCTAAAGNG, R2: TACCAGAGTATCTAATTC, R3: 5'-CTACDSRG-GTMTCTAATC and R4: 5'-TACNVGGGTATCTAATC); RDP's Pyrosequencing Pipeline: <http://pyro.cme.msu.edu/pyro/help.jsp>) were employed for PCR amplification. Molecular identifier tags were attached between the 454 adaptor sequence and the target-specific primer sequence, allowing for the identification of individual sequences from the pooled amplicons. The Ampure Purification System (Beckman Coulter) was used to clean the amplicons, before being sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Limited), in line with 454 protocols at the Teagasc high-throughput sequencing centre.

### Sequence analysis

Raw sequences were quality-trimmed and filtered, using the QIIME Suite of programs<sup>(24)</sup>. The resulting trimmed FASTA sequences were assigned to taxa through BLAST analysis, against the SILVA database (version 106) for 16S reads. BLAST outputs were parsed using MEGAN<sup>(25)</sup> with a bit-score of 86; taxonomy was assigned to phylum, family and genus levels. Sequence reads were clustered, aligned and

chimera-checked with Qiime, and phylogenetic analysis was implemented with FastTreeMP<sup>(26)</sup>. The relevant  $\alpha$ - and  $\beta$ -diversities were calculated again using Qiime, and subsequently principal coordinate analysis was performed on distance matrices. Principal coordinate analysis plots were visualised with the KiNG viewer<sup>(27)</sup>.

### Statistical analysis

To assess whether differences between treatment groups were significant, statistical analysis was performed by Student's *t* test (Graph-Pad Software). Treatment effects with  $P < 0.05$  were considered significant. Kruskal–Wallis and Mann–Whitney tests, as implemented in the SPSS statistical software package, were used to find significant differences in microbial taxa and  $\alpha$ -diversity. Significance was considered as  $P < 0.05$ . Data are presented as means with their standard errors.

## Results

### Effects of dietary *trans*-10, *cis*-12-conjugated linoleic acid on fat storage, liver weight, host metabolism and body weight

After 8 weeks of dietary supplementation with  $\text{t10c12-CLA}$ , a 2-fold decrease in visceral body fat (sum of epididymal, mesenteric and perirenal fat pads;  $P < 0.001$ ) and a significant increase in liver mass ( $P < 0.01$ ; Table 2) were found in the intervention group, compared with the unsupplemented control group. Body-weight gain and final body weight did not differ between the groups (Table 2). In addition, no difference in food intake was observed between the groups (Table 2). The greater liver weight observed in mice supplemented with  $\text{t10c12-CLA}$  was accompanied by a 7-fold increase in hepatic TAG concentrations ( $P < 0.001$ ; Table 2).

**Table 2.** Effects of *trans*-10, *cis*-12-conjugated linoleic acid ( $\text{t10c12-CLA}$ ) intake on body mass, liver mass and visceral fat mass, and on metabolic markers

(Mean values with their standard errors;  $n$  8)

	$\text{t10c12-CLA}$		Unsupplemented	
	Mean	SE	Mean	SE
Initial body weight (g)	26.7	0.8	25.4	0.5
Final body weight (g)	31.3	1.0	30.6	0.9
Body-weight gain (%)	16.0	3.0	20.5	3.1
Food intake (g)	4.5	0.1	4.3	0.1
Absolute $\text{t10c12-CLA}$ intake	0.02	0.00	–	–
Liver weight (g)	2.2**	0.1	1.5	0.1
Visceral fat weight† (g)	0.6***	0.06	1.2	0.1
Mesenteric fat weight (g)	0.4*	0.02	0.5	0.02
Perirenal fat weight (g)	0.03***	0.00	0.16	0.02
Epididymal fat weight (g)	0.16***	0.03	0.55	0.06
Serum leptin (ng/ml)	0.7*	0.2	2.4	0.7
Serum glucose (mmol/l)	11.12**	0.29	10.18	0.16
Serum insulin (ng/ml)	4.0*	0.9	1.7	0.4
Serum TAG (mmol/l)	0.32**	0.05	0.69	0.09
Liver TAG (mg/g)	20.6***	2.9	2.9	0.2

Mean value was significantly different from that of the unsupplemented control group: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ ; Student's *t* test.

† Visceral fat = sum of epididymal, perirenal and mesenteric fat pads.



In contrast, serum TAG concentrations were lower in mice fed *tl0c12*-CLA, compared with the unsupplemented control mice ( $P < 0.01$ ). Also, *tl0c12*-CLA supplementation was associated with higher serum glucose ( $P < 0.01$ ) and serum insulin ( $P < 0.05$ ) concentrations. Serum leptin concentration, which is proportional to the amount of fat in the body, was lower in the *tl0c12*-CLA-supplemented group than in the unsupplemented group ( $P < 0.05$ ; Table 2).

#### *Effects of dietary trans-10, cis-12-conjugated linoleic acid intake on host fatty acid composition*

FA profiles of the brain, epididymal adipose tissue, liver, heart and kidneys were analysed after 8 weeks of dietary *tl0c12*-CLA supplementation. The analysis revealed that *tl0c12*-CLA was incorporated into the epididymal adipose tissue, liver, heart and kidney tissues, but was not detected in the brain (Table 3). We did not detect *tl0c12*-CLA in any tissues of the unsupplemented control group. The FA composition of all tissues tested was significantly altered in the *tl0c12*-CLA-supplemented group compared with the unsupplemented control group, the greatest impact being observed on the brain, epididymal adipose tissue and liver (Table 3; Fig. 1(a)–(c)). The *tl0c12*-CLA-supplemented group had significantly higher amounts of total SFA in the brain and epididymal adipose tissue than the unsupplemented control group ( $P < 0.001$ ; Fig. 1), including a 1.6-fold increase in myristic acid (14:0;  $P < 0.05$ ) and a 1.2-fold increase in palmitic acid (16:0;  $P < 0.001$ ) in the brain, and a 2-fold increase in myristic acid ( $P < 0.01$ ) and palmitic acid ( $P < 0.001$ ) in the epididymal adipose tissue (Table 3).

Moreover, the *tl0c12*-CLA-supplemented group had significantly lower amounts of myristic acid in the heart (57%;  $P < 0.05$ ) and lower amounts of stearic acid (18:0) in the epididymal adipose tissue (17%;  $P < 0.05$ ) and liver (41%;  $P < 0.001$ ), whereas palmitic acid was higher in the liver (13%;  $P < 0.001$ ). No differences were observed in the levels of SFA in the kidney of the *tl0c12*-CLA-supplemented group compared with the unsupplemented control group. Furthermore, *tl0c12*-CLA supplementation was associated with reduced levels of MUFA in both epididymal adipose tissue and kidneys, while increased levels were found in the liver and brain (Table 3; Fig. 1). Higher amounts of palmitoleic acid (16:1-*c9*) were detected in the brain (11%;  $P < 0.05$ ) and lower amounts in the liver (31%;  $P < 0.05$ ), heart (74%;  $P < 0.05$ ) and kidneys (56%;  $P < 0.001$ ) of the *tl0c12*-CLA-supplemented group. CLA supplementation also led to increased concentrations of oleic acid (18:1-*c9*) in the liver (50%;  $P < 0.001$ ) and brain (8%;  $P < 0.01$ ), and decreased concentrations in the epididymal adipose tissue (30%;  $P < 0.01$ ) and kidneys (19%;  $P < 0.05$ ). *n-3* PUFA concentrations were also affected by *tl0c12*-CLA supplementation, with the brain of the intervention group having 24% less amounts of DHA (22:6-*n-3*;  $P < 0.001$ ), and epididymal adipose tissue having 58% less amounts of linolenic acid (18:3-*n-3*;  $P < 0.001$ ), 32% less amounts of docosapentaenoic acid (22:5-*n-3*;  $P < 0.05$ ) and 67% less amounts of DHA ( $P < 0.001$ ) than those of the unsupplemented control group. The amount of

EPA (20:5-*n-3*) was 50% lower ( $P < 0.001$ ), DHA 55% lower ( $P < 0.001$ ) and docosapentaenoic acid 33% higher ( $P < 0.05$ ) in the liver of the *tl0c12*-CLA-supplemented group compared with those of the unsupplemented control group. Moreover, the heart of the *tl0c12*-CLA-supplemented group had 57% less amounts of linolenic acid ( $P < 0.05$ ) and 60% more amounts of docosapentaenoic acid ( $P < 0.001$ ), whereas the kidneys had 43% less amounts of linolenic acid ( $P < 0.01$ ) and 45% more amounts of docosapentaenoic acid ( $P < 0.01$ ) compared with the same tissues from the unsupplemented control group.

Furthermore, significant differences in *n-6* PUFA composition were observed in the tissues of the intervention group. In the brain of these mice, arachidonic acid (20:4-*n-6*) content was decreased by 21% ( $P < 0.001$ ), while dihomo- $\gamma$ -linolenic acid (20:3-*n-6*) content was increased by 67% ( $P < 0.001$ ).

Linoleic acid (18:2-*n-6*), dihomo- $\gamma$ -linolenic acid and arachidonic acid concentrations in the epididymal adipose tissue of the intervention group were decreased by 32% ( $P < 0.001$ ), 50% ( $P < 0.001$ ) and 29% ( $P < 0.01$ ), respectively. Significantly lower amounts of  $\gamma$ -linolenic acid (18:3-*n-6*) (33%;  $P < 0.001$ ), dihomo- $\gamma$ -linolenic acid (22%;  $P < 0.001$ ) and arachidonic acid (49%;  $P < 0.001$ ) were also detected in the liver of these mice; less amounts of linoleic acid was detected in the heart (14%;  $P < 0.05$ ) and kidneys (17%;  $P < 0.05$ ), whereas higher amounts of dihomo- $\gamma$ -linolenic acid was detected in the kidneys (50%;  $P < 0.01$ ). Furthermore, the relative proportion of SFA:MUFA, an important aspect of phospholipid composition and membrane fluidity, was altered in all tissues of these mice except the heart, and the ratio of *n-6:n-3* PUFA was also changed, with higher ( $P < 0.05$ ) proportions of *n-6* PUFA in the brain, epididymal adipose tissue and liver (Table 3).

#### *Effects of trans-10, cis-12-conjugated linoleic acid intake on SCFA production*

Microbial fermentation in the caecum was enhanced by dietary supplementation with *tl0c12*-CLA. Acetate, propionate and isobutyrate levels were significantly higher ( $P < 0.05$ ) in the *tl0c12*-CLA-supplemented group than in the unsupplemented control group, whereas no difference between the two groups was observed for *n*-butyrate. Moreover, total SCFA concentration (sum of acetate, propionate, isobutyrate and *n*-butyrate) was 34% higher in the caecal content of the *tl0c12*-CLA-supplemented group than that of the unsupplemented control group ( $P < 0.05$ ; Fig. 2).

#### *Effects of dietary trans-10, cis-12-conjugated linoleic acid intake on gut microbiota composition*

A total of 416 309 V4 16S amplicon sequence reads were generated, corresponding to a mean of 23 200 reads per mouse post-quality checking. All rarefaction curves were approaching parallel (data not shown), indicating sufficient depth of sequencing. Reads were clustered into operational taxonomical units of 97% identity, and diversity metrics estimated. Of the five  $\alpha$ -diversity metrics used (Shannon,

**Table 3.** Effects of dietary *trans*-10, *cis*-12-conjugated linoleic acid (*t10c12*-CLA) intake on tissue fatty acid composition (g/100 g fatty acid methyl ester (FAME)) in mice (Mean values with their standard errors; *n* 8)

FAME	Brain				Epididymal adipose tissue				Liver				Heart				Kidney				
	<i>t10c12</i> -CLA-supplemented		Unsupplemented		<i>t10c12</i> -CLA-supplemented		Unsupplemented		<i>t10c12</i> -CLA-supplemented		Unsupplemented		<i>t10c12</i> -CLA-supplemented		Unsupplemented		<i>t10c12</i> -CLA-supplemented		Unsupplemented		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
<b>SFA</b>																					
14:0	3.1*	0.4	1.9	0.1	2.5**	0.3	1.1	0.2	0.7	0.0	0.7	0.0	0.3*	0.0	0.7	0.1	0.8	0.2	0.7	0.1	
16:0	32.9***	0.6	26.7	0.4	22.9***	1.6	10.7	0.5	34.2***	0.5	30.4	0.4	14.9	0.4	15.7	0.9	19.9	0.4	19.0	0.3	
18:0	11.2	0.1	11.1	0.2	1.9*	0.1	2.3	0.0	5.7***	0.6	9.6	0.5	15.0	0.5	13.4	1.1	13.7	0.7	12.7	0.4	
<b>MUFA</b>																					
16:1 <i>c9</i>	1.0*	0.0	0.9	0.0	2.9	0.2	2.8	0.1	1.8*	0.2	2.6	0.2	0.5*	0.1	1.9	0.5	0.7***	0.1	1.6	0.1	
18:1 <i>c9</i>	11.1**	0.2	10.2	0.1	23.4**	2.2	33.5	0.5	21.5***	1.5	10.8	0.4	9.3	0.8	12.8	1.8	11.1*	0.9	13.7	0.7	
<b><i>n</i>-3 PUFA</b>																					
18:3 <i>n</i> -3	–	–	–	–	1.1***	0.1	2.6	0.1	0.6	0.0	0.7	0.0	0.3*	0.0	0.7	0.1	0.4**	0.1	0.7	0.1	
20:5 <i>n</i> -3	–	–	–	–	–	–	0.1	0.0	0.1***	0.0	0.2	0.0	0.1	0.0	0.1	0.0	0.2	0.0	0.2	0.0	
22:5 <i>n</i> -3	0.2	0.0	0.2	0.1	0.17*	0.02	0.25	0.02	0.6*	0.1	0.4	0.0	2.5***	0.1	1.0	0.1	1.1**	0.1	0.6	0.1	
22:6 <i>n</i> -3	13.0***	0.5	17.2	0.1	0.2***	0.0	0.6	0.1	2.0***	0.2	4.5	0.2	14.4	0.6	13.2	1.4	10.2	0.7	9.3	0.8	
<b><i>n</i>-6 PUFA</b>																					
18:2 <i>n</i> -6	0.5	0.0	0.5	0.0	25.1***	1.6	36.7	0.7	17.3	0.9	19.1	0.3	19.0*	0.6	22.0	0.8	15.6*	1.1	18.9	0.7	
18:3 <i>n</i> -6	0.3	0.0	–	–	0.1	0.0	0.2	0.0	0.2***	0.0	0.3	0.0	–	–	–	–	–	–	–	–	
20:3 <i>n</i> -6	0.5***	0.0	0.3	0.0	0.2***	0.0	0.4	0.0	0.7***	0.0	0.9	0.0	0.9**	0.1	0.6	0.0	1.0	0.1	1.0	0.0	
20:4 <i>n</i> -6	4.2***	0.1	5.3	0.1	0.5**	0.0	0.7	0.0	4.0***	0.4	7.8	0.4	6.3	0.3	5.2	0.4	11.5	0.9	10.4	0.5	
<i>t10c12</i> -CLA	–	–	–	–	2.2	0.2	–	–	0.4	0.0	–	–	0.8	0.1	–	–	0.6	0.1	–	–	
<b>Ratios</b>																					
MUFA:SFA	0.26*	0.0	0.28	0.0	1.00*	0.13	2.60	0.10	0.58*	0.05	0.33	0.02	0.33	0.03	0.50	0.08	0.34*	0.03	0.48	0.03	
<i>n</i> -6: <i>n</i> -3	0.42*	0.0	0.34	0.0	17.61*	0.72	10.86	0.35	6.86*	0.24	4.84	0.09	1.52	0.07	2.00	0.21	2.43	0.19	2.92	0.23	

Mean value was significantly different from that of the unsupplemented control group: \* *P*<0.05, \*\* *P*<0.01, \*\*\* *P*<0.001; Student's *t* test.

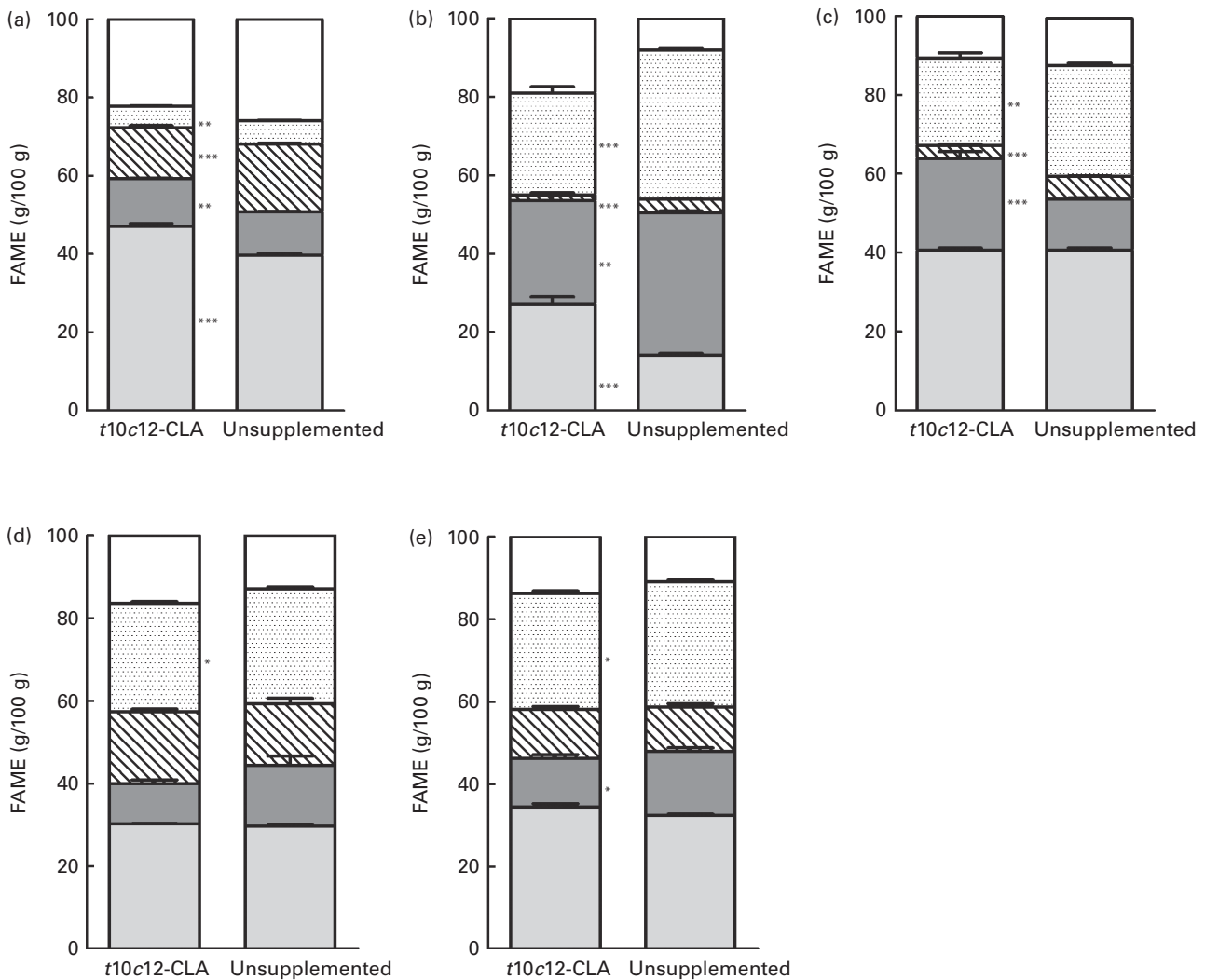
Simpson, chao1, phylogenetic diversity and observed species), no significant differences were observed between the CLA-supplemented and unsupplemented control groups. The  $\beta$ -diversity value was calculated with both weighted and unweighted Unifrac distance matrices. The subsequent principal coordinate analysis revealed a distinct separation of the two groups for both measures (Fig. 3(a) and (b)).

The mouse gut microbiota was dominated by members of the Firmicutes and Bacteroidetes phyla. Microbial composition assignment revealed several significant differences between the CLA-supplemented and the unsupplemented control groups (Fig. 4 (a) and (b)). At the phylum level, *t10c12*-CLA supplementation significantly reduced the proportions of Firmicutes ( $P=0.003$ ) and increased the amounts of Bacteroidetes ( $P=0.027$ ), when compared with no supplementation. At the family level, the members of Desulfovibrionaceae ( $P=0.027$ ), Lachnospiraceae ( $P=0.006$ ), Family XIII Incertae Sedis ( $P=0.016$ ) and Peptococcaceae ( $P=0.009$ ) were all significantly

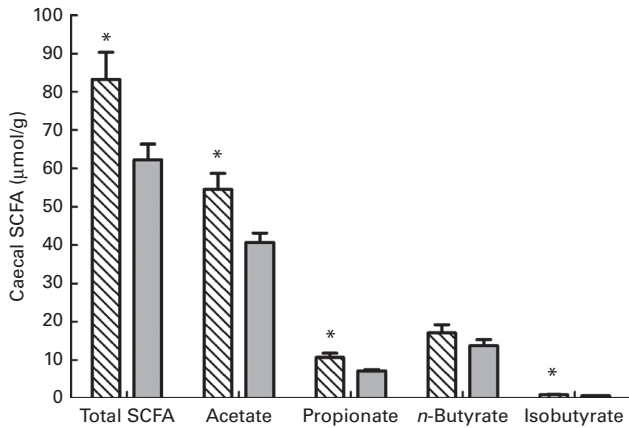
decreased in the *t10c12*-CLA-supplemented group, compared with the unsupplemented control group, while the members of Porphyromonadaceae ( $P=0.002$ ) were significantly increased in the *t10c12*-CLA-supplemented group. At the genus level, the populations of *Desulfovibrio* ( $P=0.021$ ), Lachnospiraceae Incertae Sedis ( $P=0.006$ ) and *Ruminococcus* Incertae Sedis ( $P=0.027$ ) were significantly decreased, and the populations of *Odoribacter* ( $P=0.002$ ) were significantly increased in the *t10c12*-CLA-supplemented group when compared with the unsupplemented control group.

## Discussion

The effects of dietary CLA supplementation on body fat have largely been studied in mice and, although the effects of *t10c12*-CLA seem to be independent of genetic strain<sup>(28)</sup>, the C57BL/6 mouse has been shown to be very sensitive, and it constitutes a useful model for studying lipid metabolism



**Fig. 1.** Tissue fatty acid composition in mice fed a diet supplemented with *trans*-10, *cis*-12-conjugated linoleic acid (*t10c12*-CLA) or with no supplementation. The fatty acid composition was altered by *t10c12*-CLA supplementation, with a greater impact on the (a) brain, (b) epididymal adipose tissue, (c) liver, (d) heart and (e) kidneys. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that of the unsupplemented control group (\*  $P<0.05$ , \*\*  $P<0.01$ , \*\*\*  $P<0.001$ ; Student's *t* test). FAME, fatty acid methyl esters. □, SFA; ▒, MUFA; ▨, PUFA *n*-3; ▩, PUFA *n*-6; □, others.



**Fig. 2.** Caecal SCFA concentrations in mice fed a diet supplemented with *trans*-10, *cis*-12-conjugated linoleic acid (▨) or with no supplementation (■, control group). Values are means ( $n$  8), with their standard errors represented by vertical bars. \*Mean value was significantly different from that of the unsupplemented control group ( $P < 0.05$ ; Student's  $t$  test).

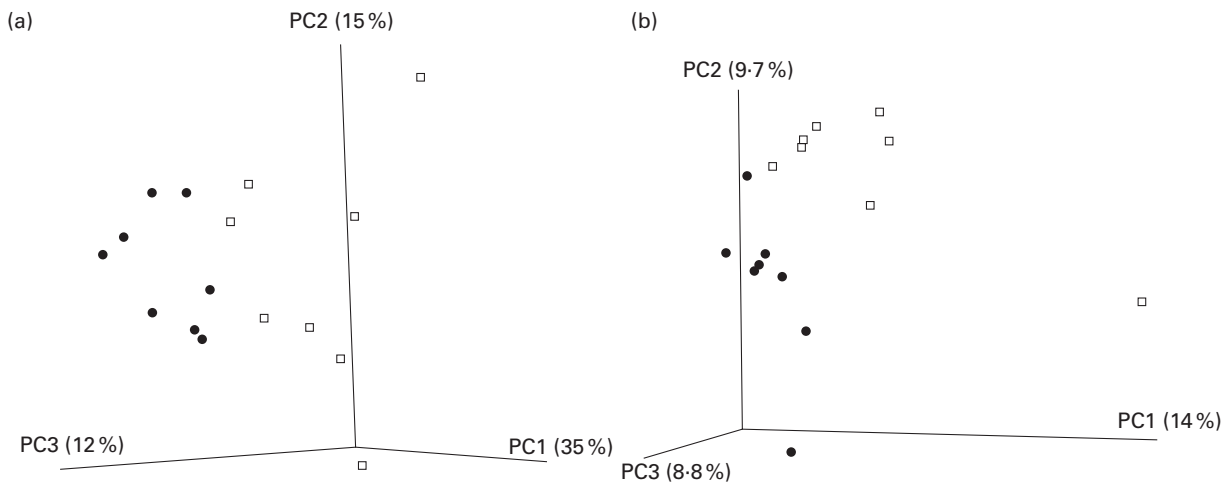
dysfunction<sup>(29)</sup>. Indeed, in the present study, C57BL/6 mice developed severe lipotrophy with concomitant liver steatosis after 8 weeks of dietary supplementation with 0.5% (w/w)  $\theta$ 10c12-CLA. Moreover, supplementation with  $\theta$ 10c12-CLA had a marked effect on the composition of the murine gut microbiota, when compared with no supplementation, despite no significant differences in diversity between groups (data not shown). Perturbations of the gut microbiota composition may play an important role in the development of diseases associated with altered metabolism such as obesity, diabetes and CVD<sup>(30)</sup>. For example, studies using germ-free animals have shown that the absence of the microbiota is accompanied by increased FA oxidation and decreased lipogenesis, making these animals resistant to diet-induced obesity, steatosis and insulin resistance<sup>(31,32)</sup>.

Furthermore, some evidence suggests that body-weight gain is associated with higher proportions of Firmicutes and lower proportions of Bacteroidetes in the gut microbiota<sup>(31,33,34)</sup>,

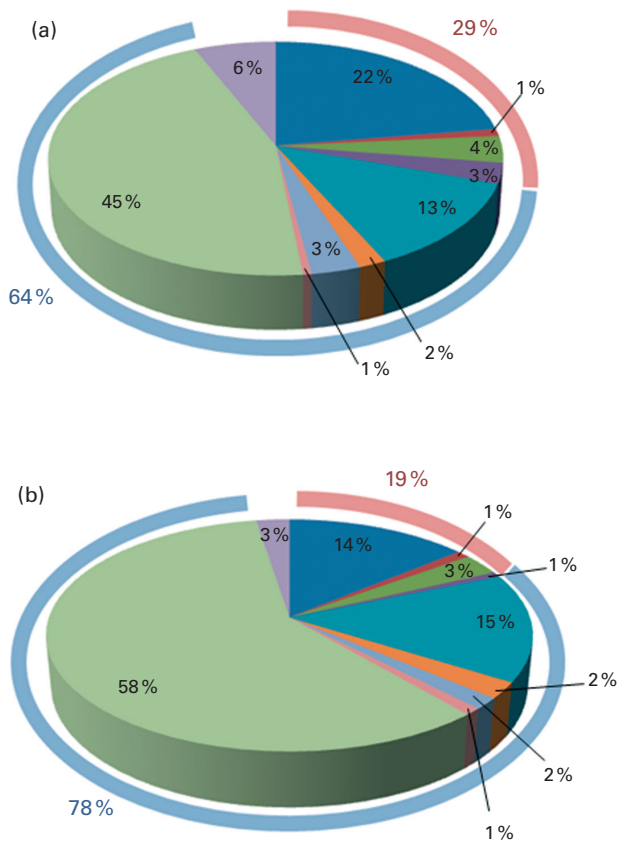
while body-weight loss has been correlated with increased abundance of Bacteroidetes<sup>(35)</sup>. In the present study, we observed that decreased body fat in mice receiving  $\theta$ 10c12-CLA was associated with higher proportions of Bacteroidetes and lower abundance of Firmicutes. Interestingly, Larsen *et al.*<sup>(36)</sup> demonstrated the same compositional changes in the intestinal microbiota of human subjects with type 2 diabetes. Moreover, they showed that a higher Bacteroidetes:Firmicutes ratio correlates positively with higher blood glucose levels and lower body mass. As type 2 diabetes is usually associated with increased body weight, these findings led to the suggestion that overweight and diabetes are associated with different groups of the intestinal microbiota, and that levels of glucose tolerance should be considered when linking the microbiota with obesity and other metabolic diseases.

Membrez *et al.*<sup>(37)</sup> further demonstrated that modulation of the gut microbiota with antibiotics influences whole-body glucose homeostasis, independent of body weight/body fat mass. In antibiotic-treated mice, reduced liver TAG levels correlated with improved insulin resistance, suggesting that the influence of the gut microbiota on glucose and liver metabolism may have similar mechanisms. The mechanism suggested for these changes was an increase in lipopolysaccharides, the main compound of the outer membrane of Gram-negative bacteria, such as bacteria from the Bacteroidetes phylum. Lipopolysaccharides have been shown to cause acute whole-body insulin resistance<sup>(38)</sup>, and are a potent stimulator of inflammation. Lipopolysaccharides activate Toll-like receptor 4 in the gut, leading to the expression of TNF- $\alpha$ <sup>(39)</sup>. Increased TNF- $\alpha$  has been shown to exert potent antiadipogenic effects<sup>(40)</sup> and induce hepatic steatosis<sup>(41)</sup>.

In a study by Le Roy *et al.*<sup>(17)</sup>, it was further confirmed that insulin resistance develops separately from obesity. However, they did not observe differences in TNF- $\alpha$  levels, and suggested that the gut microbiota may affect hepatic metabolism through other mechanisms, independent of the immune system (Fig. 5). As an example of a different mechanism, in a lipidomic study, Velagapudi *et al.*<sup>(42)</sup> suggested that the increase in phosphatidylcholine (16:0/18:1)



**Fig. 3.** Principal coordinate (PC) analysis based on (a) weighted Unifrac and (b) unweighted Unifrac distance matrices in mice ( $n$  8 per group) fed a diet supplemented with *trans*-10, *cis*-12-conjugated linoleic acid (□) or with no supplementation (●).



**Fig. 4.** Gut microbiota composition in mice fed a diet supplemented with *trans*-10, *cis*-12-conjugated linoleic acid (*t10c12*-CLA) (a) or with no supplementation (b). The microbiota composition was altered by *t10c12*-CLA intake, as determined by pyrosequencing of 16S rRNA ( $n = 8$ ). Pie charts represent the mean percentage read number for the corresponding colour-coded family (only reads  $\geq 1\%$  are shown). Bacteroidetes: ■, S24-7; ■, Bacteroidaceae; ■, Rikenellaceae; ■, Porphyromonadaceae. Firmicutes: ■, Ruminococcaceae; ■, Lactobacillaceae; ■, Erysipelotrichaceae; ■, Peptococcaceae; ■, Lachnospiraceae; ■, others.

induced by the microbiota may activate lipoprotein lipase, resulting in reduced serum TAG levels together with increased storage of lipids in the adipose tissue and liver. Although we did not examine single lipid classes in the present study, we observed an increase in 16:0 and 18:1 FA in the liver of mice fed with *t10c12*-CLA; therefore, there is a possibility that these animals could have more amounts of 16:0/18:1 phospholipids.

We also observed other important changes associated with dietary supplementation of *t10c12*-CLA. There were increased proportions of Porphyromonadaceae and decreased abundance of Lachnospiraceae and Desulfovibrionaceae. Porphyromonadaceae have previously been associated with non-alcoholic fatty liver disease, atherosclerosis and diabetes<sup>(43)</sup>. Members of the family Lachnospiraceae have been shown to protect mice against colonisation by *Clostridium difficile*<sup>(44)</sup>, whereas enhanced levels of bacteria from the Desulfovibrionaceae family were associated with impaired glucose tolerance and a serious metabolic syndrome phenotype<sup>(45)</sup>. Moreover, in a study by Bajaj *et al.*<sup>(39)</sup>, Lachnospiraceae abundance was reported to be lower in patients with cirrhosis, while

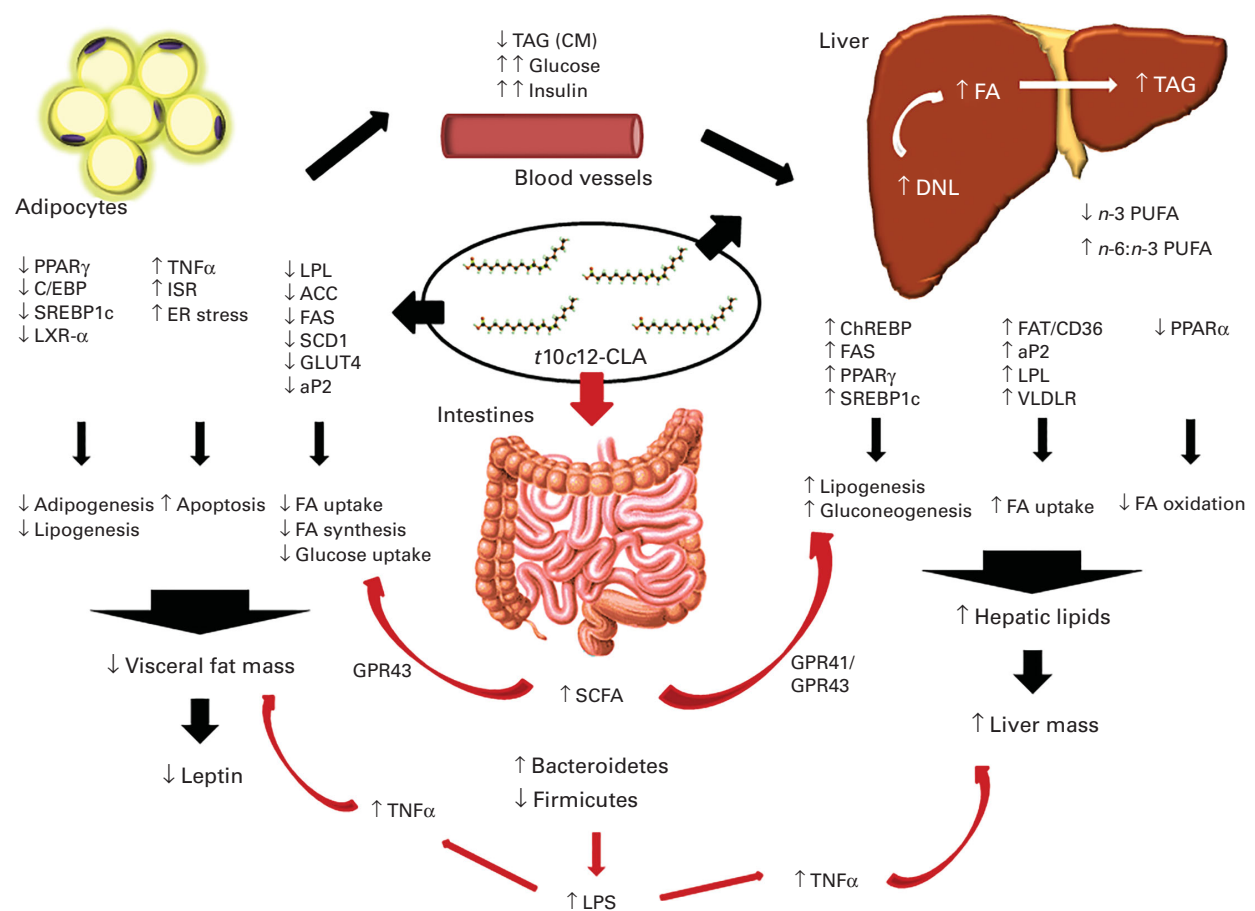
Porphyromonadaceae abundance was positively correlated with cognitive impairment and inflammation in patients with hepatic encephalopathy.

SCFA are the end products of bacterial fermentation, with acetate, propionate and butyrate being the major SCFA in the mammalian gut. The type and amount of SCFA produced depends on diet, intestinal transit and microbiota composition<sup>(46)</sup>. In the present study, SCFA levels were found to be altered by *t10c12*-CLA supplementation with higher levels of acetate, propionate and isobutyrate detected in the caecal content, even though both groups received a similar amount of carbohydrates and proteins. Thus, the higher amount of SCFA detected in the intervention group was probably due to the marked changes in the microbiota composition induced by dietary *t10c12*-CLA. SCFA are considered important energy sources for the host, and a few studies have suggested a link between increased levels of SCFA and obesity<sup>(34,47)</sup>. However, in the present study, the higher levels of SCFA were associated with lower fat mass. The precise mechanism involved in SCFA modulation of host metabolism is not clear, and the results reported in the literature are conflicting. Thus, in the present study, we propose three explanations for the possible role of SCFA in *t10c12*-CLA-induced lipotrophy/liver steatosis: (1) increased propionate induces gluconeogenesis<sup>(48)</sup>; (2) SCFA activate G-protein-coupled receptor 41, stimulating hepatic lipogenesis<sup>(49)</sup>; (3) SCFA activate G-protein-coupled receptor 43 that regulates energy uptake through the adipose tissue, and promotes the utilisation of excess energy in other tissues instead of storage in the adipocytes<sup>(50)</sup> (Fig. 5).

The fat-lowering effect of *t10c12*-CLA is complex, involving multiple mechanisms, and previous studies have shown that feeding *t10c12*-CLA triggers changes in the pattern of gene expression, reducing FA uptake and storage in the adipocytes, and favouring lipid accumulation in the liver of mice<sup>(12,19,51)</sup>. In the adipose tissue, *t10c12*-CLA has been reported to increase (pre)adipocyte apoptosis and reduce adipogenesis and lipogenesis, by the inhibition of key transcription factors<sup>(52)</sup>. The nearly complete absence of adipose tissue reduces the production of leptin by adipocytes, and the release of NEFA, thereby reducing lipid flux to the liver and subsequent VLDL secretion rates<sup>(29)</sup>. Indeed, in the present study, lower serum TAG and leptin levels were observed in the *t10c12*-CLA-supplemented group when compared with the unsupplemented control group. Moreover, *t10c12*-CLA supplementation markedly increased glucose and insulin concentrations in the serum, compared with no supplementation. Thus, *t10c12*-CLA supplementation may lead to insulin resistance and decreased glucose uptake by adipocytes, as inflammatory cytokines such as TNF- $\alpha$  and IL-6 down-regulate the expression of lipogenic proteins and the insulin-dependent GLUT 4 (for a review, see Bäckhed *et al.*<sup>(31)</sup>). High levels of circulating insulin and glucose have been reported to induce the expression of sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate response element-binding protein in the liver, and, therefore, stimulate hepatic *de novo* lipogenesis<sup>(53,54)</sup>.

Another factor that may contribute to the development of liver steatosis is the profound change in the composition





**Fig. 5.** Schematic summary of all the mechanisms of action of *trans*-10, *cis*-12-conjugated linoleic acid (*t10c12*-CLA), as proposed by the different studies cited in the present paper. There are a variety of proposed mechanisms through which *t10c12*-CLA supplementation may cause hepatic steatosis and lipotrophy in mice. Also, *t10c12*-CLA up- and down-regulates genes involved in fatty acid (FA) synthesis, uptake and oxidation in the adipose tissue and liver in a direct and an indirect manner (black arrows). In the present study, we propose that the gut microbiota and its products are extra environmental factors affecting host lipid metabolism (red arrows). ACC, acetyl-CoA carboxylase; aP2, adipocyte-specific fatty acid binding protein; C/EBP, CAAT/enhancer-binding protein; ChREBP, carbohydrate response element binding protein; CM, chylomicron; DNL, *de novo* lipogenesis; ER, endoplasmic reticulum; FAS, fatty acid synthase; FAT/CD36, fatty acid translocase; GPR, G-protein coupled receptor; ISR, integrated stress response; LPL, lipoprotein lipase; LPS, lipopolysaccharide; LXR- $\alpha$ , liver X receptor- $\alpha$ ; SCD1, stearoyl-CoA desaturase; SREBP-1c, sterol regulatory element-binding protein 1c; VLDLR, VLDL receptor.

of long-chain fatty acids in the liver, induced by *t10c12*-CLA supplementation. The present study showed that *t10c12*-CLA intake was associated with an increased *n*-6 PUFA:*n*-3 PUFA ratio in the liver. Reduced availability of *n*-3 PUFA may increase SREBP-1 expression, and reduce PPAR $\alpha$  expression, stimulating lipogenesis over lipid oxidation, with accumulation of TAG in the liver<sup>(55)</sup>. Furthermore, *t10c12*-CLA supplementation was associated with altered FA composition of other tissues, besides changing liver FA profiles. Our observations confirmed previous reports demonstrating that *t10c12*-CLA supplementation decreased arachidonic acid, and altered the ratio of SFA:MUFA, especially increasing the amount of palmitic acid over palmitoleic acid<sup>(6,28,56)</sup>. Although the exact mechanism of CLA action on tissue FA composition has not been elucidated, a few studies have suggested that the reduction in stearoyl-CoA desaturase activity may impair the conversion of SFA to MUFA<sup>(28,35)</sup>, whereas the inhibition of linoleic acid elongation and desaturation may have an impact on *n*-6 PUFA synthesis<sup>(57,58)</sup>.

Several studies investigating the anti-obesity effect of CLA supplements have focused on gene regulation in the liver

and adipose tissue; however, to our knowledge, this is the first study to show the impact of dietary *t10c12*-CLA on the gut microbiota composition and SCFA production. Long dietary exposure to *t10c12*-CLA transformed the gut microbiota, favouring the growth of harmful bacteria, thus increasing host susceptibility to a variety of diseases. The greater proportions of Bacteroidetes and Porphyromonadaceae bacteria found in the *t10c12*-CLA-supplemented group most probably had an influence on lipid metabolism and induction of hepatic steatosis, with higher levels of SCFA contributing to enhanced lipogenesis and gluconeogenesis in the liver.

Thus, diet plays an important role in the modulation of the gut microbiota composition, and as observed in the present study, a single dietary FA is capable of inducing a systemic effect on the host. Although data on the effects of CLA in human subjects are contradictory, few studies that used purified isomers instead of CLA mixtures have shown a detrimental influence of *t10c12*-CLA, raising concerns about the safety of supplements containing higher amounts of this isomer<sup>(59,60)</sup>. Considering the fact that non-prescription pills

for weight loss are frequently used as an easier alternative to diet and physical activities<sup>(61)</sup>, more clinical studies of longer duration with larger sample sizes are needed.

Dietary approaches targeting beneficial bacteria and suppressing harmful species may be a new strategy to prevent or treat hepatic steatosis and associated metabolic disorders. The use of FA mixtures with equal proportions of CLA isomers, or the use of probiotics and prebiotics to balance the potentially negative effects of t10c12-CLA on the microbiota composition may be alternatives for individuals seeking anti-obesity dietary solutions.

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The authors' responsibilities are as follows: C. S., R. P. R., F. S. and E. M. Q. designed the research; T. M. M. and R. W. conducted the research; T. M. M., R. W. and O. O. analysed the data; T. M. M., R. W., O. O. and C. S. wrote the manuscript; G. F. F., P. D. C., T. G. D. and J. F. C. provided significant advice; C. S. had primary responsibility for the final content.

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