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Yvette Bouton [a], Raymond Berges [b], Stéphanie Cabaret [b], Eric Beuvier [c], Lionel Bretillon [b], Olivier Berdeaux [b]*

[a] *Comité Interprofessionnel de Gestion du Comté, R&D Unit, F-39801, Poligny, France*

[b] *Centre des Sciences du Goût et de l'Alimentation, CNRS, INRA, Université Bourgogne Franche Comté, Agrosup Dijon, F-21000, Dijon, France.*

[c] *URTAL, INRA, F-39801, Poligny, France*

*Corresponding author. Tel.: +33 (0)380 693 540

E-mail address: olivier.berdeaux@inra.fr (O. Berdeaux)

ABSTRACT

The study was designed to compare the effect of diets of equal calcium and fat content from pressed-cooked cheese, butter, palm oil or a chow diet, on blood lipids, faecal fat excretion and body composition in rats. Palm oil intake resulted in higher faecal saturated fatty acid (SFAs) excretion compared with pressed-cooked cheese or butter, showing the importance of the positional distribution of SFAs in triacylglycerol. However, compared with butter, cheese fat intake resulted in higher faecal fat excretion, confirming the importance of the dairy-matrix effect in the excretion of fat. Despite a high consumption of fat, animals of pressed-cooked cheese group did not gain weight (no differences in any groups) and did not accumulate fat compared with the control group, contrary to the situation with rats fed with the same amount of fat provided by vegetable oil and extracted from butter that gained fat mass compared with controls.

1. Introduction

Similar to animal-derived fats and in particular dairy products, cheeses such as Comté are rich in lipids, and are a significant source of saturated fatty acids (SFAs) in the human diet (SFAs represent approximately 65 % of the total fatty acids). However, SFAs suffer from a negative image in health. Their consumption is associated with the development of cardiovascular diseases (CVD) mainly linked to the increase of serum cholesterol concentration (Keys et al., 1986). Nevertheless, recent studies do not show a negative effect of cheese intake on cholesterolaemia or metabolic syndrome (De Goede, Geleijnse, Ding, & Soedamah-Muthu, 2015; Nilsen, Torbjorn Hostmark, Haug, & Skeie, 2015; Soerensen, Thorning, Astrup, Kristensen, & Lorenzen, 2014).

A possible explanation for this discrepancy could be due to the high content of calcium in dairy products. Fatty acids with long chains released during the hydrolysis of dietary triacylglycerols form insoluble soaps with calcium that promote their excretion in faeces and thereby reduce their intestinal absorption (Gueguen & Pointillard, 2000; Lorenzen et al., 2007). However, whether calcium influences the absorption of lipids depends on the structure of the triacylglycerols. Indeed, on the one hand only fatty acids hydrolysed from positions *sn*-1 and *sn*-3 of triacylglycerols form soaps at the intestinal level and can be excreted by this way (Raynal-Ljutovac et al., 2011). On the other hand, the accessibility of the enzyme to its substrates is modulated by the physical structure of milk fat that may in turn modify its efficacy (Ayala-Bribiesca, Lussier, Chabot, Turgeon, & Britten, 2016).

The objective of the present study was to establish whether the bioavailability of dietary SFAs from pressed-cooked cheese is different from that of vegetable oil or butter. For this, we compared the effects of four diets of equal fat content (8% of lipids) composed of a mixture of vegetable oils alone or supplemented with either palm oil, pressed-cooked cheese or butter, on blood lipids, faecal fat excretion and body composition in rats. A control chow

diet with half-fat content (4% of lipids) of the same mixture of vegetable oils was also tested.

2. Materials and methods

2.1. Chemical products

HPLC-grade n-hexane, chloroform and methanol were purchased from Fisher Scientific (Illkirch, France). Chemical reagents, boron trifluoride in 14% methanol (BF3-MeOH), Heptadecanoic acid (C17:0) and Supelco® 37 component fatty acid methyl esters (FAME) mix were obtained from Sigma Aldrich (Saint Quentin Fallavier, France).

2.2. Preparation of pressed-cooked cheese

Pressed-cooked cheese was produced in 100 L copper vat with raw milk at the mini experimental cheesemaking plant of the Institut National de la Recherche Agronomique (INRA, URTAL, Poligny, France). Milk was inoculated with 0.12% *Streptococcus thermophilus* starter culture and, 0.08% *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *lactis* starter culture. The scalding temperature was 55 °C. Cheese was ripened for 6 months (4 weeks at 12 °C, 5 weeks at 16 °C, and 17 weeks at 6 °C). Gross and microbiological compositions of ripened cheese (see details below) were determined according to Bouton, Buchin, Duboz, Pochet, and Beuvier (2009) and Bertrand et al (2007), respectively.

Physico-chemical composition per 100 g of ripened cheese was as follows: dry matter, 66.8 g; dry fat matter, 53.2 g; sodium chloride, 0.9 g; calcium, 1.3 g. Microbiological composition per g of ripened cheese was as follows: presumptive thermophilic streptococci, 3.2×10^5 cfu; presumptive thermophilic lactobacilli, 7.3×10^6 cfu; mesophilic lactobacilli, 2.4

× 10^7 cfu; propionic acid bacteria, 5.4 × 10^5 cfu.

2.3. Animals

All procedures were performed in agreement with the regulations from the French Ministry of Research and after approval by the local ethic committee of the University of Burgundy (C2EA Grand Campus Dijon N° 105). A total of forty 6-week-old specific pathogen free (SPF) male Wistar Han rats were purchased from Harlan France sarl (Gannat, France). They were acclimatised to the animal facility conditions (22 °C room temperature with 55% relative humidity and a 12 light: 12 dark period) for one week. Each rat was housed individually in a polycarbonate cage. During the acclimatisation week, all rats were provided with a 4% lipids purified diet and were supplied with filtered tap water ad libitum.

2.4. Diets and study design

At day d0, rats were randomly assigned to each of the five experimental groups (G1, G2, G3, G4 and G5) (n = 8 per group).

Rats were fed for 30 days with different purified diets (INRA, Jouy en Josas, France) containing (g kg^{-1}) casein 180, cornstarch 400 (G1, G3) or 380 (G2, G4 and G5), maltodextrin 210 (G1, G3) or 190 (G2, G4 and G5), sucrose 60, cellulose 50, mineral mixture 50, vitamin mixture 10 (see Supplementary material) and lipids 40 (G1) or 80 (G2, G3, G4 and G5).

Lipids composition was as follows: control group (G1) was fed with a purified diet containing 4% by weight of basic oil mixture obtained by mixing colza, palm, oleic sunflower and sunflower oils, (18.5:38.25:38.25:5, by wt). High fat control group (G2) was fed a purified diet containing 8% by weight of basic oil mixture. Cheese experimental group (G3) was fed with a purified diet containing 4% by weight of basic oil mixture and in addition at

the same time, received daily a piece (5 g) of pressed cooked cheese. This amount of cheese was chosen so that the total lipid intake was close to 8% by weight. Vegetable oil experimental group (G4) was fed a purified diet containing 4% by weight of basic oil mixture and 4% by weight of palm oil. Butter experimental group (G5) was fed a purified diet containing 4% by weight of basic oil mixture and 4% by weight of butter. All rats were supplied with filtered tap water ad libitum. Total dietary amount of calcium calculated was 7962 mg (G1, G2 and G4), 8027 mg (G3) or 7977 mg (G5).

Body weight (BW) and food consumption were recorded once a week. The amount of food consumed in grams per day was converted in kcal and expressed per day as a function of body weight.

2.5. Sample collection and euthanasia

Twelve hours before collecting faeces, rats were placed in clean cages with a grid in the bottom to collect faeces (2 g). Blood (0.5 mL) was collected by tail vein puncture. Faeces and blood samples were collected from individual rats at days 0, 8, 15, and 30 (d0, d8, d15, and d30). Erythrocytes were separated from plasma by centrifugation at 3000 rpm for 10 min at +4 °C then stored like faeces at 80 °C. At d31 all the animals were killed after overnight fasting by CO_2 inhalation and perirenal white adipose tissue was collected and stored at –80 °C until lipid analysis.

2.6. Distribution of FA in triacylglycerols

The position of FA in triacylglycerol molecules of pressed-cooked cheese, butter or palm oil, have been determined by gas liquid chromatography (GC) analysis before and after hydrolysis by pancreatic lipase and purification of the 2-monoacylglycerols by thin layer

chromatography according Kuksis (1984).

2.7. Analyses of fatty acids

Lipids were extracted from adipose tissue following the procedure described by Folch, Lees, and Sloane Stanley (1957) whereas lipids were isolated from plasma according to the method of Moilanen and Nikkari (1981). All lipid extracts were stored at –80 °C until lipid analysis.

Lipids of dry faecal matter were hydrolysed and extracted according to the method described by Toullec, Flanzy, and Rigaud. (1968). Briefly, about 1 g of dry faeces were weighed and introduced in a tube containing internal standard (300 µL of an heptadecanoic acid (17:0) solution at 10 mg mL^{-1}) for the quantification of fatty acids. Ten millilitres of a solution containing ethanol, water and concentrated HCl (5:4:1, by vol) were added and after 30 min, the mixture was homogenised for 2.5 min using an Ultra turrax homogeniser (Fisher Scientific, Illkirch, France). Hexane, 12.5 mL, was then added and the mixture homogenised again for 2.5 min. The emulsion was centrifuged (4000 × *g*, 5 min) and organic phase was collected and transferred into a separatory funnel. Three successive homogenisations and extractions were required for each sample. The hexane solution was washed with water until neutrality. Residual water was eliminated by separative filtration through anhydrous sodium sulphate using Whatman No. 1 filter paper (Sigma Aldrich, Saint Quentin Fallavier, France). After hexane evaporation, the hydrolysed lipids were cold-saponified overnight in 5 mL of an alcohol solution of 10% potassium hydroxide. 5 mL of water was then added. Fatty acids were extracted after acidification of the aqueous phase using hexane (3 × 10 mL) as a solvent. After washing and further elimination of traces of water, the solvent was evaporated under a stream of nitrogen and the fatty acid content was gravimetrically determined.

Prior to gas chromatography analysis, total fatty acids from plasmas, adipose tissue

and faeces were transmethylated using BF3-MeOH according to Morrison and Smith (1964). FAMEs and dimethylacetals (DMAs) were subsequently extracted with hexane and analysed by gas chromatography on a Hewlett Packard Model 5890 gas chromatograph (Palo Alto, CA, USA) using a CPSIL-88 column (100 m × 0.25 mm i.d., film thickness 0.20 µm; Varian, Les Ulis, France) equipped with a flame ionisation detector. Hydrogen was used as the carrier gas (inlet pressure 210 kPa). The oven temperature was held at 60 °C for 5 min, increased to 165 °C at 15 °C min^{-1} and held for 1 min, and then to 225 °C at 2 °C min^{-1} and finally held at 225 °C for 17 min. The injector and the detector were maintained at 250 °C. FAMEs and DMAs were identified by comparison with commercial and synthetic standards. The data were processed using the EZChrom Elite software (Agilent Technologies, Massy, France) and reported as a percentage of the total fatty acids.

2.8. Echo-MRI measurement

One day prior to euthanasia, body composition was measured using Echo-MRI systems (Echo Medical Systems, Houston, TX, USA) as described previously (Thierry et al., 2014). Fat mass and lean mass were calculated as the percentage of total body weight.

2.9. Statistical analyses

The data were analysed using SAS software (SAS Institute, Cary, NC, USA). Pearson's correlation coefficients were calculated to determine associations between tissue profiles. Associations were considered significant at a level of $P < 0.05$. Repeated measures ANOVA were used to investigate how body weight of the rats and cumulative lipid intake were structured with time. Analyses were performed using R Studio (version 3.1.1 2014) available at https://www.rstudio.com.

3. Results

3.1. Fatty acids composition of purified diets

Table 1 presents the total fatty acid composition of the different diets (G1, G2, G3, G4 and G5) used for this study. Experimental diets containing pressed-cooked cheese G3, butter G5 and palm oil G4 contained equivalent amounts of medium and long chain saturated fatty acids (14:0 to 24:0): about 38 %. G3 and G5 diets contained 4 % short-chain saturated fatty acids and about 3 % of trans fatty acids (vaccenic acid: 18:1 Δ11t, and rumenic acid: 18:2 Δ9c,11t) while the vegetable oil did not contain short 162 chain fatty acids, and only 0.6 % of trans fatty acids (elaidic acid: 18:1 Δ9t). In the different diets, the final ratio ω6/ω3 was between 4.6 and 8.7.

3.2. Lipid and food intake of animal

The cumulative daily food intake showed no significant differences in any groups (data not shown). But taking into account that the caloric densities of the diets were different (4 Kcal g^{-1} of diet in G1 and G3, 4.2 Kcal g^{-1} in G2 and G4 and 3.98 Kcal g^{-1} in G5), the total amount of lipids consumed, based on feed intake measurement, was significantly higher in groups G2 to G5 than in group G1, with the highest amount being observed in group G3 (Fig. 1A). Fig. 1B presents the cumulative caloric intake of the rats, relative to body weight from week 1 to week 5, indicating that group G2 and G5 had a significant increased caloric intake from week 2 to week 5, compared with others. Interestingly animals from the group G3 that consumed 5 g of pressed-cooked cheese daily and preferably before the chow intake had the same caloric intake as rats from the control group G1.

3.3. Lipid composition of butter, pressed-cooked cheese and palm oil

Lipids in butter, pressed-cooked cheese and vegetable oils were prominently triacylglycerols. The fatty acid composition of triacylglycerols and distribution of fatty acids in the 3 positions of glycerol in pressed-cooked cheese, butter and palm oil are presented in Table 2. As expected the pressed-cooked cheese and butter presented the same fatty acid composition. SFAs represented 66% of total fatty acids (mainly myristic acid, palmitic acid and stearic acid) and unsaturated fatty acids represented 33% (mainly oleic acid and linoleic acid). Palm oil had a fatty acid composition in which the level of saturated fatty acids (53%) was almost equal to unsaturated fatty acids (46%).

The fatty acid distribution within the three moieties of glycerol in triacylglycerols from pressed-cooked cheese was very close to that of butter. SFAs are mainly in external position (62%) against (38%) in internal position. By contrast, SFAs were mainly esterified in external positions (84%) of glycerol in palm oil, compared with the internal position (16%).

3.4. Animal body weight

As reported in Fig. 2, no significant differences in body weight of the animals were observed between the different groups considering the overall diet time from day 0 to day 30.

3.5. Body composition of animals

The results of body mass are presented in Table 3. Fat mass was calculated as the percentage of total body weight. It was significantly increased in groups G2 and G5, compared with control group G1. Lean mass was significantly decreased only in group 5. Next, the ratio between fat mass and lean mass was found to show a significant increase in groups G2 and G5 compared with control group G1. It is interesting to note that animals from

group G3 who were fed with cheese, have the same ratio (0.18) than control group G1.

3.6. Fatty acid composition in erythrocytes, plasma, adipose tissue and faeces of animals at d30

Fig. 3A–D shows the percentage distribution of total SFAs, monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs) and *trans* fatty acids in erythrocytes, plasma, adipose tissue and faeces at day 30. Despite a greater intake of SFAs in groups G3, G4 and G5 and therefore lower intake of MUFAs and PUFAs compared with G1 (Table 3), there was no significant differences between groups in the fatty acid composition of plasma and erythrocytes (Fig. 3A,B). Small amounts of *trans* fatty acids were detected in rats fed with lipids of animal origin (G3 and G5), and only traces in plasma of rats from group G4. It is interesting to note a lower but significant accumulation of SFAs (34%) in adipose tissue of animals of group G4 than in groups G3 and G5 (37%) (Fig. 3C). Finally, adipose tissue of animals fed with diet containing dairy lipids (G3 and G5), contain non-negligible amount of *trans* fatty acids.

Analyses of fatty acid in faeces showed that faeces of animal in groups G2, G3 and G5 contained the same percentage of SFAs (78–80%) whereas the faeces of the animals from G1 group contained less (69%) and those of animals from G4 contained more (83%) (Fig. 3D). In addition, to Fig. 3D, Fig. 4 shows the amount of fatty acids (mg g^{-1} of dry faeces) eliminated in the faeces of rats, calculated after adding of an internal standard. It is important to note that the rats in batch G3 and G4 eliminated 3.5 and 4.7 times more fatty acids, respectively, in their faeces than rats of control group G1. In other hand, rats in group G2 and G5 eliminated only 1.5 and 2 times more fatty acids than the rats of the control group. The fatty acids in the faeces of rats in groups G3 and G4 were mainly composed of SFAs, suggesting that a part of the SFAs consumed was eliminated via the faeces. It can be deduced that a bigger part of

SFAs consumed from diets G3 and G4 were eliminated via the faeces compared with G5 diet, which nevertheless contain the same amount of SFAs, or even compared with control diet G2.

4. Discussion

The objective of this study was to evaluate whether a diet enriched in fat from pressed-cooked cheese modifies blood lipids, faecal fat excretion and body composition in rats compared with a diet containing a similar fat content extracted from butter or palm oil. The reason for comparing pressed-cooked cheese and butter is that they present a similar fatty acid composition and a similar positional distribution of fatty acids on glycerol as the pressed-cooked cheese, although butter contains little calcium (15 mg 100 g^{-1} in butter versus 940 mg 100 g^{-1} in pressed-cooked cheese such as Comté on average). However, in our study, due to higher calcium content in mineral mixture, diets contained (per kg diet) 7.96 g (G1, G2, G4,), 7.98 g (G5) or 8.03 g (G3). The rationale for comparing pressed-cooked cheese and palm oil is that they exhibited a similar SFA content, but differ from the position of these fatty acids on glycerol, with 80% unsaturated fatty acids and only 20% SFAs in sn-2 position according to Renaud, Ruff, and Pettihory (1995). The positioning of the fatty acids on the glycerol moieties is biologically fundamental since it regulates the mechanism of the intestinal absorption of the fatty acids (Bracco, 1994; Mattson, Granville, Nolen, & Marjorie, 1979).

The influence of calcium on the absorption of lipids is highly dependent on the structure of the triacylglycerol, because only acylglycerols hydrolysed in position sn-1 and sn-3 are likely to form soaps at the intestinal level and be eliminated in faeces (Mattson et al., 1979). Our results obtained with animals fed with vegetable oil (experimental group G4) confirm that, despite significant amounts of SFAs in palm oil, they are not well absorbed and they are essentially eliminated in faeces. According to previous studies, this is particularly due

to their positions on triacylglycerols essentially in sn-1 and sn-3 positions and making them less bioavailable (Kayden, Senior, & Matsson, 1967; Mattson et al., 1979; Small 1991). However, faeces of animals in experimental group G3 fed with pressed-cooked cheese also contained high levels of SFAs. Although diets G3 and G5 have the same fatty acid composition and the same distribution of FAs on glycerol, animals in the G3 group absorbed less SFAs than animals from lot G5. Actually, the levels of FAs found in the faeces of animals of group G3 are almost twice as high as those found in the faeces of the animals of group G5. These results are consistent with a previous study in pigs showing a significantly higher fat excretion on the diet with cheese than on the diet with butter (Thorning et al., 2016a). The chemical environment (fermentation, minerals such as calcium, protein, etc.) of fatty acids in fermented cheeses determines their intestinal absorption.

Finally, although animals of the group G3 (pressed-cooked cheese) consumed more fat than animals of G2, G4 and G5, these animals did not gain more weight and it is interesting to note that they did not tend to accumulate fat unlike animals of G2 and G5 treatments. It is important to notice that the rise in body fat mass was reported only in animals fed with 8% of vegetable oil (G2) or with 4% of vegetable oil plus 4% of fat extracted from butter (G5). On the contrary, animals fed with the same 8% amount of fat as the form of 4% of vegetable oil plus a piece of cheese that provided 4% of fat (G3) did not gain fat mass. This was in agreement with meta-analyses suggesting that intake of milk and dairy products was associated with reduced risk of childhood obesity (Thorning et al, 2016b).

5. Conclusion

Despite the high proportion of SFAs in pressed-cooked cheese, the balanced distribution of SFAs between the sn-2 and sn1/sn-3 positions of glycerol and the impact of dairy-matrix fat content were associated to a lower degree of absorption, and greater faecal

excretion.

These effects on lipid could be of interest for the release of fat-soluble nutrients or bioactive molecules especially at the moment of their passage into the gastrointestinal tract (Ayala-Bribiesca, Turgeonand, & Britten, 2017). This work shows the effects of different diets on lipids absorption and faecal excretion. It seems important to consider the whole of dairy matrix in further research on the health effects.

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Fig. 1.

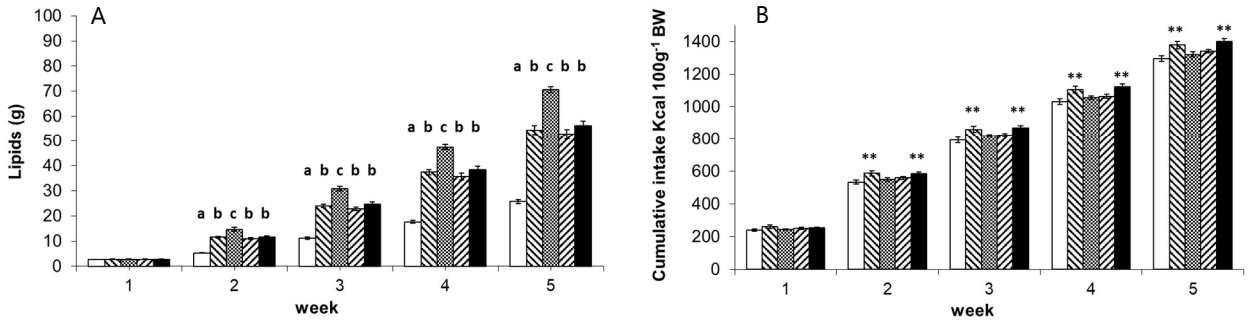
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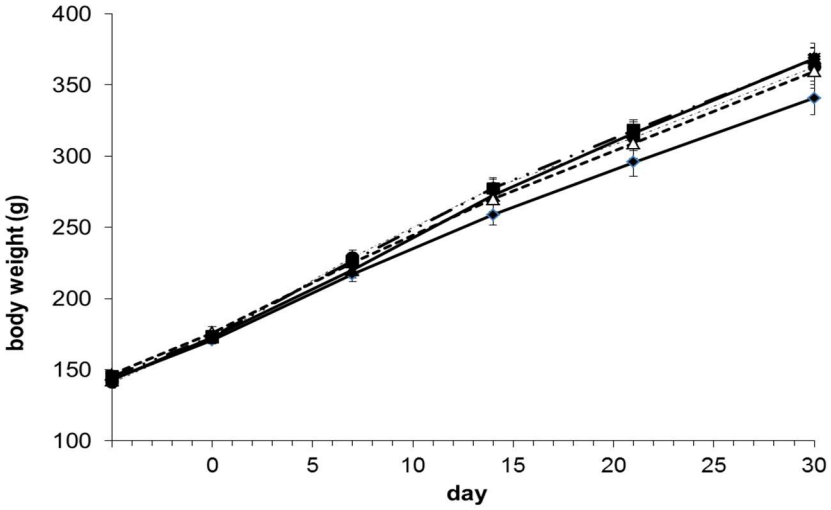
Fig. 2.



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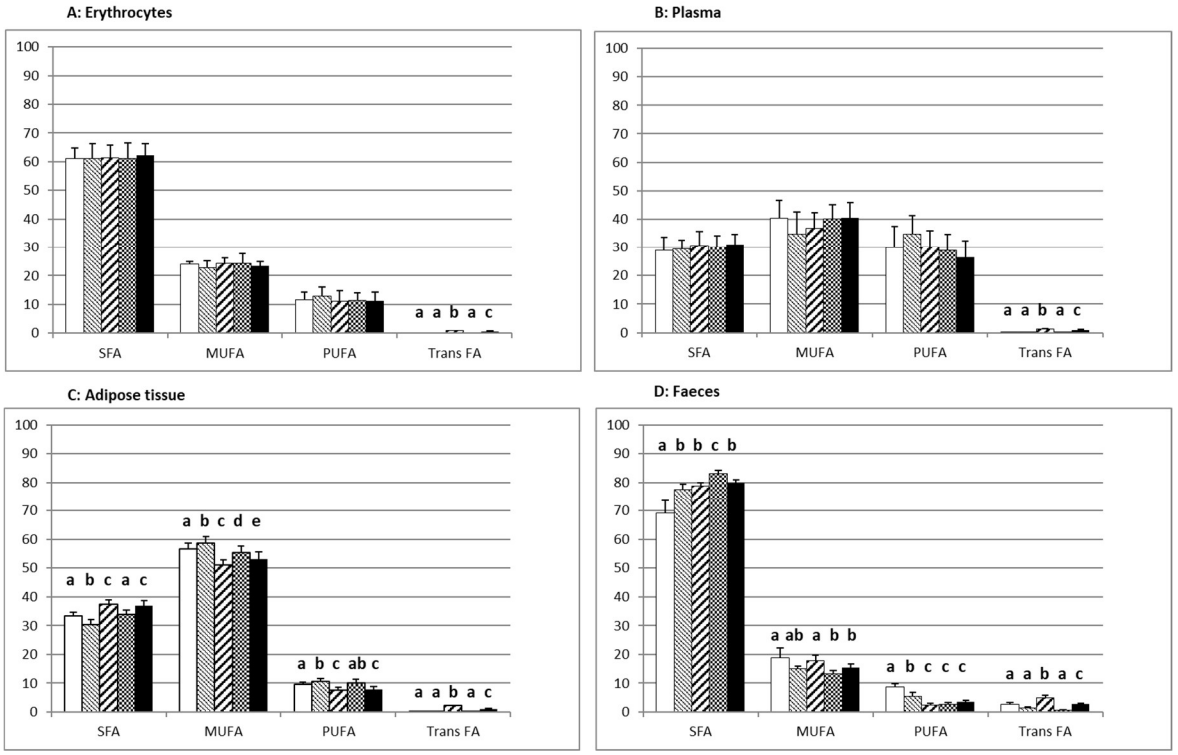
A: Erythrocytes
B: Plasma
C: Adipose tissue
D: Faeces
SFA
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Fig.4.

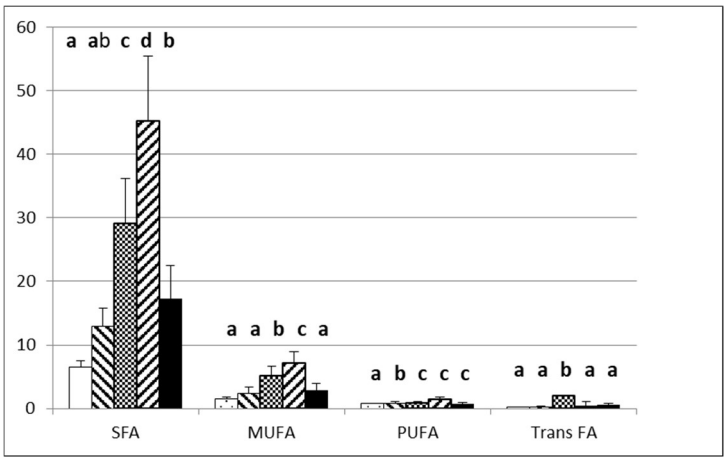
60
50
40
30
20
10
0
a ab c d b
a a b c a
a b c c c
a a b a a
SFA
MUFA
PUFA
Trans FA

Table 1

Amount of total lipids and fatty acid composition in different purified diets G1, G2, G3, G4 and G5. [a]

Fatty acids	Fatty acid composition in diet (g 100 g^{-1} of lipids)				
	G1	G2	G3	G4	G5
14:0	0.9	0.6	5.6	0.9	5.5
16:0	19.4	19.3	22.8	31.6	23.2
16:1ω7	0.2	0.2	0.7	0.2	0.7
17:0	0.1	0.1	0.4	0.1	0.4
18:0	3.6	3.5	6.7	4.0	7.0
18:1t	0.2	0.2	2.0	0.2	2.0
18:1 ω 9	55.6	56.7	42.1	47.4	41.7
18:1 ω 7	1.7	1.7	1.1	1.4	1.1
18:2 ω 6	13.7	13.1	8.3	11.1	8.1
20:0	0.4	0.4	0.3	0.4	0.3
20:1 ω 9	0.5	0.5	0.3	0.3	0.3
18:3 ω 3	2.4	2.4	1.8	1.3	1.8
18:2 9c,11t (CLA)	0.0	0.0	0.7	0.0	0.6
22:0	0.5	0.5	0.4	0.3	0.4
24:0	0.2	0.2	0.2	0.1	0.2
Others	0.6	0.6	6.7	0.7	6.6

[a] Abbreviation: CLA, conjugated linolenic acid. Total lipids in diet (mg g^{-1} of diet) were: G1, 41.0; G2, 80.0; G3, 72.0; G4, 79.0; G5, 76.0.

Table 2

Fatty acid composition of triacylglycerols and fatty acid position on glycerol (g 100 g^{-1} total fatty acids) of pressed-cooked cheese, butter and palm oil. [a]

Fatty acid	Total FA composition of TAG			FA position location: sn-2 / sn-1 and sn-3		
	P-CC	Butter	Palm oil	P-CC	Butter	Palm oil
12:0	3.6	3.3	0.2	44 / 56	47 / 53	14 / 86
14:0	12.5	12.1	1.0	54 / 46	55 / 45	24 / 76
15:0	2.0	2.5		48 / 52	47 / 53	
16:0	28.7	29.0	45.1	36 / 64	35 / 65	17 / 83
16:1	1.6	1.6	0.2	45 / 55	45 / 55	24 / 76
17:0	1.3	1.4		32 / 68	25 / 75	
18:0	11.4	11.8	5.8	15 / 85	14 / 86	12 / 88
18:1	26.5	27.5	38.0	23 / 77	23 / 77	50 / 50
18:2	3.9	3.1	7.6	27 / 73	28 / 72	63 / 37
20:0	0.2	0.2	0.4	14 / 86	14 / 86	11 / 89
SCFAs	5.9	5.6		28 / 72	27 / 73	
SFAs	59.7	60.2	52.7	38 / 62	37 / 63	16 / 84
MUFAs	28.2	29.3	38.3	25 / 75	24 / 76	50 / 50
PUFAs	4.8	3.9	7.7	25 / 75	27 / 73	62 / 38

[a] Abbreviations are: FA, fatty acid; TAG, triacylglycerol; PCC, pressed-cooked cheese; SCFAs, short chain fatty acids; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.

Table 3

Body composition (at day 30) of rats fed with different purified diets G1, G2, G3, G4 or G5 for 30 days. [a]

Diet	Fat mass/BW (%)	Lean mass/BW (%)	Fat mass/lean mass
G1	13.40 ± 0.77	73.33 ± 0.68	0.18 ± 0.01
G2	16.35 ± 1.18*	72.80 ± 1.03	0.23 ± 0.02*
G3	13.79 ± 0.98	75.54 ± 0.88	0.18 ± 0.02
G4	15.07 ± 1.20	73.94 ± 1.17	0.21 ± 0.02
G5	18.16 ± 1.79*	71.00 ± 1.60*	0.26 ± 0.03*

[a] Data are means ± SEM; an asterisk indicates significant difference to G1 ($P < 0.05$) as analysed by Mann Whitney test.