



Preliminary data on the dietary safety, tolerability and effects on lipid metabolism of the marine microalga *Tisochrysis lutea*

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ARTICLE INFO

Keywords:

Safety
Microalgae
Food
Tisochrysis lutea
Nutraceutical
Lipid metabolism

ABSTRACT

The marine microalga *Tisochrysis lutea* is an interesting source of nutrients and bioactive compounds such as fucoxanthin and docosahexaenoic acid, used so far mainly in aquaculture. To investigate its dietary safety and tolerability on mammals, male Sprague-Dowley rats were fed an AIN-76 diet containing 20% of *T. lutea* F&M-M36 biomass, for 1 month.

T. lutea rich diet showed an apparent digestibility similar to that of the non enriched AIN-76 diet and did not affect growth or animal behavior, but was associated to higher water intake, urinary excretion and urinary sodium probably due to the high salt content of the algal biomass. However, blood pressure, creatinine and urea, kidney morphology and heart left ventricular wall thickness were not affected. *T. lutea* fed rats showed an increase in cholesterol high density lipoprotein, HDL ($p < 0.05$) and decreased plasma triglycerides ($p = 0.06$), together with an increased excretion of fecal lipids ($p < 0.05$). Up-regulation of PPAR γ ($p < 0.05$) and UCP-1 ($p < 0.05$) and down-regulation of LPL genes ($p < 0.05$) in the liver of *T. lutea* fed rats were also observed.

These preliminary data indicate that the *T. lutea*-rich diet was well tolerated in the short term and suggest that this marine microalga may represent a promising source of functional foods and bioactive compounds for the control of dyslipidemias. Its salt content, however, poses a safety issue, which must be overcome before proposing its use in humans.

1. Introduction

Tisochrysis lutea El M. Benif & I. Probert [1] is a marine microalga belonging to the Haptophyceae, originally isolated from tropical seawater (Tahiti, French Polynesia). Although *T. lutea* is currently used mainly in aquaculture, its high content of protein and fibers, together with the presence of several bioactive compounds, makes it an interesting source of nutraceutical and pharmaceutical products [2]. *T. lutea* is in fact rich in polyunsaturated fatty acids (PUFA), mainly docosahexaenoic acid (DHA, C22:6 ω 3) [3,4], and carotenoids such as fucoxanthin [5].

T. lutea is not commercially available for human consumption unlike other microalgae such as *Chlorella*, *Dunaliella*, *Arthrospira*, *Nostoc*, *Aphanizomenon* and *Tetraselmis* [6,7] and its safety need still to be evaluated.

In a preliminary screening in human cells and in *Artemia salina*, we

recently observed that *T. lutea* F&M-M36 extracts exhibit an IC50 of 6 g/L, showing an intermediate degree of toxicity compared to the other strains analyzed [6].

Nuno et al. (2013) observed no acute toxicity in rats fed *I. galbana* T-ISO (= *Tisochrysis lutea*) up to 50 mg/day. In the same study, the microalga administered at a dosage of 50 mg/day for 8 weeks promoted body weight loss in healthy rats and maintained the weight in those with diabetes; neither significant histopathological alterations of the gastrointestinal tract nor kidney function impairment were reported in healthy rats, but diabetic rats exhibited some indication of superficial intestinal chronic low-inflammation [9]. Herrero and coworkers (1993) conducted a study administering *I. galbana* Parke, a microalga phylogenetically close and physiologically similar to *T. lutea*, to weaning rats as the sole source of protein, corresponding to a 35% of algal biomass in the diet, for four weeks [8]. Compared to a casein treated group, decreased weight gain and a higher intake of water were observed.

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Relative heart weight was also lower, but no haematological abnormalities with the exception of increased blood urea, were reported. Besides genetics, one of the main differences between *I. galbana* and *T. lutea* is that *I. galbana* is rich in both eicosapentaenoic acid (EPA, C20:5 ω 3) and DHA, while *T. lutea* only in DHA (Molina Grima et al., 1992; Bendif et al., 2013; Ryckebosch et al., 2014; Tibaldi et al., 2015). These few studies on rats were not intended to specifically address the dietary safety and tolerability at high dosages. Therefore, we performed an extensive study by testing the effects of a diet containing 20% *T. lutea*, administered for 1 month to healthy rats. This percentage corresponds to 12 g of microalgal dry biomass/kg of body weight, translatable into a daily intake of 159 g in a 70 kg human, by applying the human equivalent dose (HED) calculation [10], thus, much above the expected daily human consumption.

2. Material and methods

2.1. Biomass production, preparation and composition

T. lutea F&M-M36, from the Fotosintetica & Microbiologica S.r.l. Culture Collection of Microalgae and Cyanobacteria, was cultivated in GWP® photobioreactors [11] in a semi-batch mode. The total sodium concentration in the medium (30 g/L salinity) was 0.37 mol/L. The biomass was harvested by a centrifugal separator (Westfalia mod. SSD18, GEA Group Aktiengesellschaft, Düsseldorf, Germany), frozen, lyophilized and powdered. Before lyophilization the dry biomass content in the concentrate was 12–15%. The powdered biomass was stored at -20°C until use. Total protein content was estimated as $N \times 6.25$, where N is the nitrogen content determined through elemental analysis (CHNSO Analyzer, Thermoelectron Corp., USA). Carbohydrate content was determined following Dubois et al. (1956) and lipid content following Marsh & Weinstein (1966) [12,13]. Humidity was analyzed following ISTISAN protocols (ISTISAN Report 1996/34, Method B, Page 7). Fiber was determined according to AOAC Method 985.29. Fatty acid composition was evaluated by using the standard method for food of the Italian Ministry for Health (ISTISAN Report 1996/34, p. 47). Fatty acids were extracted from lyophilized biomasses and methylated. The methyl esters were analyzed with a GC–MS system (Abiusi et al., 2014). Fatty acids were identified by comparing retention times with those of authentic standards (Supelco® 37 Component FAME Mix, Italy). Sodium was determined by atomic absorption spectrometry (Rupérez, 2002). The content of salt was estimated from sodium concentration multiplied by 2.5 (He et al., 2014). DNA and RNA were extracted from the freeze-dried algal biomass by using TRIzol (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions and quantified by using a Nano-Photometer UV/Vis Spectrophotometer (Implen GmbH, München, Germany).

2.2. Fucoxanthin determination

The fucoxanthin content of *T. lutea* F&M-M36 biomass and of the abdominal fat in *T. lutea* F&M-M36 fed rats was studied. Sudan Red (Sigma–Aldrich, Germany) (270 μL), the monitoring standard for UV–Vis, and β -apo-carotenol (Sigma–Aldrich, Germany) (150 μL), the internal standard for quantification, were added to *T. lutea* F&M-M36 freeze-dried biomass or to the fat (20 mg). A methanol solution (7.5 mL) was added and the solutions were heated at 60°C for 15 min. A diethyl ether/petroleum ether solution (50:50, 7.5 mL) and a NaCl solution (20% in water, 5 mL) were added and the solutions were carefully stirred. The upper phase was collected in a rotary evaporator flask, dried and then resuspended with a methanol/methyl tertiary butyl ether (MTBE) 4:1 and butylated hydroxytoluene (BHT) (0.01%) solution (3 mL). Chromatographic analysis of extracts from *T. lutea* F&M-M36 biomass and from fat was carried out according to a modification of the method by Kim et al. (2012). Fucoxanthin separation was achieved with an HPLC (Hewlett Packard 1050, California, USA)

equipped with a C30 reverse phase column (YCM Carotenoid, 4.6 mm \times 250 mm, 5 μm particle size) (Waters, Massachusetts, USA), and a UV photodiode array detector (Hewlett Packard 1050, California, USA). A gradient method with two eluents was used, eluent A: 81% MTBE, 10% methanol, and 9% deionised water and eluent B: 93% MTBE and 7% methanol. The injection volume was 20 μL with a constant flow rate of 1 mL/min, at 25°C temperature. The detection was performed at 450 nm. The quantification was performed by internal standard calibration. Commercial fucoxanthin (Sigma–Aldrich, Germany) standard solutions (20, 40, 60, 80, 100, 120 $\mu\text{g mL}^{-1}$ in methanol/MTBE 4:1), with β -apo-carotenol (50 $\mu\text{g mL}^{-1}$) and Sudan Red (90 $\mu\text{g mL}^{-1}$) were prepared. The rate between the area under the peaks of fucoxanthin standard solutions and the area under the internal standard peak was plotted against fucoxanthin standard solution concentrations ($\mu\text{g mL}^{-1}$) to obtain a calibration curve adopted to quantify the concentration of fucoxanthin in the microalgal biomass and in the fat sample [5].

2.3. Diets preparation

The AIN-76 (American Institute of Nutrition, 1977) diet was prepared from its components (Laboratorio Dottori Piccioni S.r.l., Milan, Italy) and contained 5% fat (corn oil). In the microalga-rich diet, which contained 20% lyophilized microalgal biomass, the different components were adjusted so as to compensate for protein, lipid, carbohydrate and fiber deriving from *T. lutea* F&M-M36 biomass and to maintain the caloric intake of the diet (Table 1).

2.4. Animals and treatments

All procedures were carried out in agreement with the European Union Regulations on the Care and Use of Laboratory Animals (OJ of ECL 358/1, 12/18/1986), according to Italian regulations on the protection of animals used for experimental and other scientific purposes (DM 116/1992), after approval from the Italian Ministry for Scientific Research. We used 6- to 8-weeks male Sprague-Dowley rats (Nossan S.r.l., Milan, Italy). The animals were housed in plastic cages with wire tops and maintained at a temperature of 22°C , with a 12:12-h light-dark cycle. After their arrival from the supplier, animals were acclimatized for a week, during which they were fed a standard lab chow. Rats were then randomly allocated to two experimental groups: rats fed AIN-76 diet (controls, $n = 4$) or a *T. lutea* F&M-M36 rich diet ($n = 8$), ad libitum, for 1 month.

Individual animal body weights were recorded weekly, starting from the first day of experiment. During the third week of treatment, the animals were placed in metabolic cages for one day in order to collect 24-h urine and feces, to assess the apparent digestibility of the diet and to measure water daily consumption. Samples of feed and fecal samples were collected and weighed and oven-dried at 55°C until constant weight. After drying, the coefficient of apparent digestibility

Table 1
Composition of the experimental diets (g/100 g of diet).

	AIN-76 diet	<i>T. lutea</i> F&M-M36 rich diet
Lyophilized algal biomass	–	20
Corn oil	5	2
Sucrose	50	50
Starch	15	11.8
Casein	20	11.5
Cellulose	5	1.4
Mineral Mix AIN 76	3.5	3.5
Vitamin Mix AIN 76	1	1
Coline	0.2	0.2
DL Methionine	0.3	0.3

Values in bold indicate constituents of the diet that were adjusted in order to compensate for components deriving from algal biomass.

was calculated as follows: Apparent digestibility % = [feed dry weight – 24-h feces dry weight/feed dry weight] × 100.

Systolic, diastolic blood pressure, mean arterial pressure (MAP) and frequency were monitored in conscious rats by noninvasive computerized tail-cuff method (Visitech BP-2000 Series II Blood Pressure Analysis System) at week 3 of treatment, after two days of training.

At the end of the study, rats were euthanized by inhalation of CO₂. External surface, all orifices and the cranial, thoracic, abdominal and pelvic cavities, including viscera of all rats were examined. Brain, heart, kidneys, liver, bladder, caecum, colon, spleen were collected, weighed and stored for further analyses.

The thickness of the tibio-tarsal joint was also measured by using a micrometer (screw-gauge) to assess the possible presence of peripheral edemas. Blood was collected for clinical biochemistry (lipid profile, kidney and liver function).

2.5. Blood and urine biochemistry

Plasma chemistry parameters were performed at Careggi Hospital, Florence, Italy and included: urea, creatinine, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, low density lipoprotein (LDL) and high density lipoprotein (HDL). Urinary sodium and uric acid were also measured and expressed relative to the 24 h urine.

2.6. Histological analysis

For histological evaluations, tissue samples (heart, kidney, liver, urinary bladder and brain) were collected and fixed in phosphate buffered 4% formalin for 12 h. Samples were dehydrated in ethanol and embedded in paraffin. 5 μm thick sections were hematoxylin-eosin stained and observed for morphological analysis. Regarding heart, the thickness of the ventricular wall was also measured. In detail, five microscopical fields per animal were registered by a digitizing camera applied to a light microscope with a 20 x objective, each field corresponding to a test area of 141,100 μm². On digitized images, measurements of ventricular wall thickness were carried out using ImageJ 1.33 image analysis software (<http://rsb.info.nih.gov/ij>) by two independent observers in blind fashion.

2.7. Fecal water and lipid content

Fresh fecal samples were harvested and frozen at –20 °C until analyses. To determine the degree of diarrhea, fecal samples were dried in a dehumidified oven at 50 °C until stable in weight and the water content was expressed as percentage of the fresh fecal weight [14]. To estimate lipid content, dried fecal samples (about 30 mg) were re-suspended in 500 μL of normal saline. Then, 500 μL of chloroform-methanol (2:1; v/v) were added in order to extract the lipids, according to <http://www.bio-protocol.org/e1375>.

2.8. RT-PCR

RNA was extracted from tissue homogenates by using a commercially available kit following manufacturer's instructions (Macherey-Nagel, Bethlehem, USA). For first-strand cDNA synthesis, 1 μg of total RNA from each sample was reverse-transcribed by using the RevertAid RT Kit (Thermo Scientific, Waltham, MA USA). Primers were designed on the basis of the rat GenBank sequences (Supplementary Table 1). For each target gene, the relative amount of mRNA in the samples was calculated as the ratio of each gene to β-actin mRNA [15].

2.9. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5.0 software (GraphPad, San Diego, CA). D'Agostino and Pearson omnibus

Table 2

Fatty acid composition of *T. lutea* F&M-M36 biomass (% of dry weight).

Fatty acids composition	% of dry weight
SFA	7.10
MUFA	3.80
PUFA	4.14
22:6n-3 (DHA)	1.01
20:5n-3 (EPA)	< 0.1
18:3n-3 (α-linolenic acid)	1.60
Total ω-3	2.61
18:2n-6c (linoleic acid)	1.13
18:3n-6 (γ-linolenic acid)	0.04
20:2n-6 (eicosadienoic acid)	< 0.1
20:3n-6 (eicosatrienoic acid)	0.35
20:4n-6 (arachidonic acid)	< 0.1
Total ω-6	1.17
18:1n-9c (oleic acid)	2.91
Total ω-9	2.91
ω-6/ω-3	0.45

SFA: Saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.

normality test was applied to verify the Gaussian distribution of each variable. Differences on body weight gain, organs weight, clinical chemistry parameters, fecal water and lipid content, blood pressure measurements and on gene expression were analyzed using unpaired *t*-test with Mann Whitney correction or *t*-test when appropriate. Results are presented as means ± SEM. Significance was assigned at *p* < 0.05.

3. Results and discussion

Thanks to their huge biodiversity, microalgae are a fascinating source of biologically active compounds with potential application as nutraceutical or pharmaceutical products [16]. In this context, the marine microalga *T. lutea* F&M-M36 deserves particular attention as it combines a relatively high content of proteins (42.4%) and fibers (18.2%) and with a very good content of mono- (3.8%) and polyunsaturated (4.1%) fatty acids, including DHA (Table 2), and of the carotenoid fucoxanthin (0.6%), whereas ash content was 13.1%.

To our knowledge, this is the first study addressing the safety and tolerability of a *T. lutea* F&M-M36-rich diet in rats.

During the study period, no adverse effects regarding food consumption, animal behavior or physical abnormalities were observed with the alga-enriched diet. The growth curves and weight gain were not statistically different from those of the controls by Mann-Whitney test (Supplementary Fig. 1).

Given that this observation is limited to the 30 days feeding period, we cannot exclude significant effects in the long term, as reported by Nuno et al. (2013) and Herrero et al. (1993) [8,9].

The apparent digestibility of the *T. lutea* F&M-M36 rich diet was slightly lower compared to the AIN-76 diet (– 4%) but the difference of caecum weight was not significant between the two groups. However, the amount of daily feces and the percentage of fecal water content were significantly augmented (*p* < 0.05), suggesting that *T. lutea* F&M-M36 may also exert positive effects on intestinal health, which deserves to be better explored (Table 3). The *T. lutea* F&M-M36 rich diet contains more NaCl compared to the AIN76 normal salt diet (1.5% vs 0.3%); this is very likely the reason for the double water daily consumption (Table 3), as also previously reported by Herrero et al. [9], triple urine daily production (Table 3) and significantly increased 24 h urinary Na⁺ excretion compared to controls (5.7 ± 0.45 vs 0.48 ± 0.05 mE in 24 h; *p* < 0.01).

A very high salt diet (8% of NaCl) causes left ventricular hypertrophy and elevates blood pressure [17]; in our experiment, we observed an increased relative heart weight in rats fed *T. lutea* F&M-M36 (*p* < 0.05; (Supplementary Table 2) that was neither associated to an increased left ventricular wall thickness, a sign of cardiac hypertrophy,

Table 3Effects of *T. lutea* F&M-M36-rich diet on water daily consumption, urine and feces production and on fecal water content.

	Water daily consumption (ml)	Urine daily production (ml)	Feces daily production (g)	Water fecal content (%)	Fecal lipids excretion (%)
AIN-76 diet # n = 4	21.5 ± 1.7	7.0 ± 1.3	1.8 ± 0.4	50.4 ± 8.9	13.7 ± 2.5
<i>T. lutea</i> F&M-M36 diet = 8	42.0 ± 1.9 **	21.6 ± 1.1 **	3.8 ± 0.4 *	64.5 ± 1.1*	24.7 ± 3.5

Data are expressed as mean ± SEM. *p < 0.05; ** p < 0.01. (# data relative to AIN-76 control diet, were previously published elsewhere, [19]).

Table 4Effect of *T. lutea* F&M-M36-rich diet on biochemical and blood pressure parameters.

	AIN-76 diet # n = 4	<i>T. lutea</i> F&M-M36 diet n = 8
Urea g/L	0.4 ± 0.0	0.3 ± 0.0
Creatinine mg/dL	0.3 ± 0.1	0.3 ± 0.0
AST U/L	290.5 ± 176.5	180.0 ± 67.5
ALT U/L	75.0 ± 16.1	59.2 ± 4.5
Alkaline phosphatase U/L	155.0 ± 28.0	173.7 ± 12.9
Triglycerides mg/dL	187.062.0	52.3 ± 5.2 (p = 0.06)
Total cholesterol mg/dL	73.0 ± 4.0	131.5 ± 6.2*
HDL mg/dL	55.0 ± 5.3	117.9 ± 5.8*
LDL mg/dL	5.0 ± 0.0	7.0 ± 1.3
Systolic	142.9 ± 5.3	130.2 ± 7.6
Diastolic	81.1 ± 7.1	66.8 ± 14.4
Pulse	386.1 ± 32.3	351.3 ± 27.2

nor to blood pressure variations (Table 4) or peripheral edemas. Creatinine and urea levels, measured to assess renal function variations (Table 4), and kidney histopathology were also normal. However, since excessive salt contributes to cardiovascular risk, a reduction of NaCl content of the algal biomass, for instance by modifying the composition of the growth medium or by adopting a more efficient washing, is necessary before *T. lutea* F&M-M36 can be proposed in humans.

MAP = mean arterial pressure. Data are expressed as mean ± SEM; *p < 0.05. (# data relative to AIN-76 control diet, were previously published elsewhere, [19]).

Issues associated with the use of microalgae as a food source, also include their nucleic acid content, which upon metabolism produce uric acid leading to diseases such as gout and uric acid nephrolithiasis. The content of DNA and RNA of the *T. lutea* F&M-M36 biomass was 0.1% and 0.8%, respectively, resulting in an intake of 120 mg/kg of nucleic acids per day. By applying HED calculation [10] the daily dose in rats translates into 1.5 g/day in a 70 kg human, below the safety limit of 4 g/day established by the World Health Organization [18]. Nevertheless, the 24 h urinary uric acid excretion, even if within the values previously reported for control rats [17], was augmented in *T. lutea* F&M-M36 group compared to controls (0.07 ± 0.01 vs 0.15 ± 0.01 mg; p < 0.01); moreover, in the kidney, in the proximal and distal convoluted tubules of a single rat fed *T. lutea* F&M-M36, we observed the presence of an amorphous and eosinophilic deposit (Supplementary Fig. 2).

The kidneys of remaining animals did not show any morphological damage but in the same animal, a huge crystal and amorphous deposit, macroscopically similar to a little stone, rolling in the urinary bladder was found. An analogous formation was described in our previous study focused on an *Arthrospira platensis* Gomont-rich diet [19]. Although this may be caused by retrograde seminal vesicles discharges, we cannot exclude the deposition of an uric acid crystal [20]. Even if the dose of *T. lutea* F&M-M36 in the 20% diet largely exceeds the expected human consumption, these findings, although sporadic, should not be ignored when proposing its use in humans.

Despite a reduced weight (p < 0.05) (Supplementary Table 2), the liver showed a normal morphological structure in both experimental groups. In hepatocytes, no cyto-pathological signs of steatosis, hemosiderin accumulation or biliary stasis were present (data not shown). Serum aspartate transaminase (AST), alkaline phosphatase and alanine

aminotransferase (ALT) did not indicate any hepatic impairment (Table 4).

Interestingly, although rats were all fed a well-balanced and isocaloric diet, we observed unexpected, positive effects on lipid metabolism in animals fed the alga-enriched diet. Compared to the control group, total cholesterol concentration was significantly higher, but this effect was mainly ascribed to the augmented HDL (+112%) (p < 0.05) since LDL levels did not change (Table 4). Rats fed *T. lutea* F&M-M36 showed also significantly higher fecal excretion of lipids (+75%) (Table 2), suggestive of a decreased intestinal lipid absorption, which resulted in reduced plasma triglycerides (−73%) (p = 0.06) (Table 4).

Reduced triglycerides levels were reported by Herrero and coworkers (1993) in weaning rats fed *I. galbana*, but they did not investigate the mechanism involved [8]. On the contrary, Nuno et al. (2013) reported no effects on triglycerides using *I. galbana* T-ISO (*T. lutea*) as supplement [9]. Various bioactive components in *T. lutea* F&M-M36, including ω-3 fatty acids and fucoxanthin, might act synergistically to alter plasma lipid profile and fecal fats concentration. ω-3 have long been known to dose-dependently lower plasma triglycerides with the minimal effective dose being > 2 g/day [21]. In our study, we calculated that rats received about 30 mg of DHA daily, corresponding to a daily intake of 1.58 g in a 70 kg human. *T. lutea* biomass also contains ω-6 (about 10.1% of total fatty acids) but the ratio ω-6/ω-3 in this microalga strain is favorable (0.58) since a low ω-6/ω-3 ratio beneficially modulates the lipid profile in rats [22]. A fucoxanthin-supplemented diet (0.2%) increased plasma HDL and fecal excretions of total lipids, reducing total cholesterol and triglycerides in mice fed a high fat diet [23]. *T. lutea* F&M-M36 biomass contains 0.6% (dry weight) of the carotenoid fucoxanthin corresponding to 0.12% in the diet, but no trace of fucoxanthin in the abdominal fat (despite being intensely orange colored) of *T. lutea* F&M-M36 fed rats was detected maybe due to its rapid biotransformation into fucoxanthinol and amarouciaxanthin A or to a very low level of fucoxanthin accumulation in the adipose tissue [24].

However, since we observed similar positive results on lipid profile in rats fed an *A. platensis* (spirulina) rich diet, a microalga with different fatty acid and carotenoid compositions [19], we cannot exclude that other bioactive compounds may have a role. Some studies have in fact suggested that dietary proteins with low ratios of methionine–glycine and lysine–arginine, such as those from soy, compared to casein, exert also hypocholesterolemic and hypotriglyceridemic effects in experimental animals [25]. Both *A. platensis* biomass used in our previous study [19] and *I. galbana* T-ISO (*T. lutea*) [26], have low ratios of methionine–glycine (0.08 and 0.37 vs 0.97) and lysine–arginine (0.56 and 0.98 vs 2.4), compared to casein [27], the sole source of proteins in the AIN-76 diet. It is worth considering that the beneficial effects on blood lipids of the *T. lutea* F&M-M36 rich diet, were observed despite that only about half of the total protein content was from the microalgal biomass and the remaining from casein (Table 1).

To explore the possible molecular mechanisms involved in the beneficial effects on lipid profiles, we analyzed the expression of PPAR-α, PPAR-γ, HMGCR, APOA-1, UCP-1, LPL and APOCIII genes in the liver. A hierarchical cluster analysis distinguished the different expression profiles of these genes in the liver of rats fed *T. lutea* F&M-M36 and in controls (Fig. 1).

Interestingly, we observed an increased expression of PPAR-γ and UCP-1 and a reduced expression of LPL in the liver of *T. lutea* F&M-

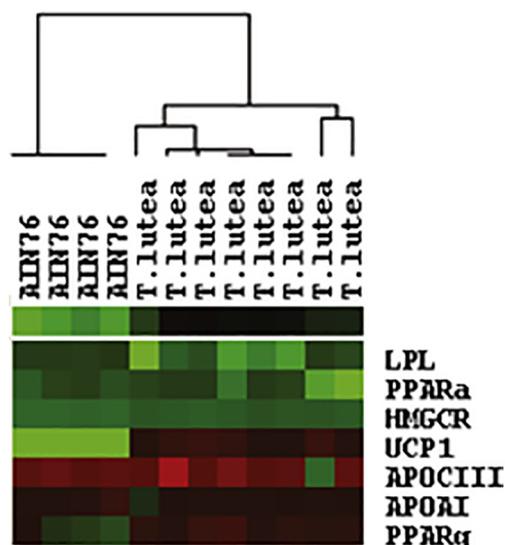


Fig. 1. Hierarchical cluster analysis of the liver gene expression profiles of rats fed *T. lutea* F&M-M36-rich diet or the AIN-76 control diet. Each column represents a different rat and each row a different gene; the color code indicates down-regulation (green) or up-regulation (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5

Expression of PPAR- α , PPAR- γ , HMGCR, APOA-1, UCP-1, LPL and APOCIII in the liver.

	AIN-76 diet n = 4	<i>T. lutea</i> F&M-M36 diet n = 8
PPAR- α	0.52 \pm 0.04 #	0.46 \pm 0.07
PPAR- γ	0.76 \pm 0.16	1.51 \pm 0.08 *
HMGCR	0.38 \pm 0.005	0.43 \pm 0.006
APOA-1	1.33 \pm 0.13	1.07 \pm 0.03
UCP-1	0.04 \pm 0.003	1.48 \pm 0.10 *
LPL	0.63 \pm 0.01	0.37 \pm 0.06 *
APOCIII	2.8 \pm 0.07	2.7 \pm 0.41

Data are expressed as mean \pm SEM of the relative amount of each gene vs the housekeeping gene, b-actin. *p < 0.05. (# data relative to AIN-76 control diet, were previously published elsewhere, [19]).

M36-fed animals compared to controls (p < 0.05) (Table 5).

Although PPAR- γ is expressed at a much lower level in the liver than in adipose tissue, PPAR- γ dependent effects in the liver have been reported. The brown alga wakame (*Undaria pinnatifida*), also containing fucoxanthin, increased PPAR γ expression in the liver and ameliorated lipid profiles in a murine model of diet-induced obesity [28], augmenting also the expression of UCP-1 gene, usually expressed only in adipose tissue [29]. The very low levels of plasma triglycerides observed in *T. lutea* F&M-M36 fed rats could be associated also to the unexpected increased expression of UCP-1 in the liver. The ectopic up-regulation of UCP-1, in fact, decreased body weight and reduced fat in the liver and adipose tissues in high fat diet-induced dyslipidemia [30]. LPL is a gene target of PPAR- γ signaling and its liver-specific over-expression increased liver triglyceride content and insulin resistance in mice [31]. These effects are of interest since PPAR γ agonist, such as thiazolidinediones, improve dyslipidaemia but are not devoid of side effects.

4. Conclusions

Overall, our results show that a balanced diet, supplemented with 20% *T. lutea* F&M-M36 biomass is well tolerated over 30 days and does not elicit significant negative effects in rats, but the relatively high salt

content of the biomass represents a safety concern to be addressed before being used as food; nucleic acid content may also limit the amount of algal biomass that can be included in the diet. Indeed, we observed that T-ISO biomass added 5% in the diet did not increase water consumption or diuresis and did not affect uric acid excretion in a long-term experiment in rodents (manuscript in preparation). Despite the small number of animals used in the present study that may limit the significance of our results, we observed that lipid metabolism was significantly and positively affected by *T. lutea* F&M-M36-based diet. These preliminary effects on lipid homeostasis and on PPAR- γ signaling deserve to be better explored in a model of metabolic syndrome or dyslipidemia where the biological relevance of these findings could be highlighted.

Statement of informed consent, human/animal rights

The authors wish to declare that there was no conflict, informed consent, human or animal rights applicable. Rats were handled in agreement with the European Union Regulations on the Care and Use of Laboratory Animals (OJ of ECL 358/1, 12/18/1986), according to Italian regulations on the protection of animals used for experimental and other scientific purposes (DM 116/1992), after approval from the Italian Ministry for Scientific Research (1137/2015-PR).

Declaration of authors' agreement to authorship and submission of the manuscript for peer review

The authors wish to declare our agreement to authorship and submission of the manuscript for peer review.

Author contribution

CL, MRT: study conception and design; CL: obtained funding as per acknowledgements; EB, LC, MDA, ML and AN: animal treatment, histopathology, gene expression; MRT, AN, LR, NB: microalgae cultivation and characterization; MDO: fucoxanthin determination; EB and CL: data analysis and manuscript drafting. All the authors participated in discussing the results and in revising the article.

Conflict of interest

T. lutea F&M-M36 belongs to the F&M S.r.l. culture collection. M.R. Tredici and L. Rodolfi have a financial interest in F&M S.r.l. The other authors have no conflicts of interest.

Acknowledgements

This research was supported by a Grant from Ente Cassa di Risparmio Firenze (N°2015.0919).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2018.08.008>.

References

- [1] E. Bendif, I. Probert, D.C. Schroeder, C. de Vargas, On the description of *Tisochrysis lutea* gen. nov., sp. nov. and *Isochrysis nuda* sp. nov. in the Isochrysidales, and the transfer of *Dicrateria* to the Prymnesiales (Haptophyta), *J. Appl. Phycol.* 25 (2013) 1763–1776.
- [2] L. Custódio, F. Soares, H. Pereira, L. Barreira, C. Vizetto-Duarte, M.J. Rodrigues, A.P. Rauter, F. Albericio, J. Varela, Fatty acid composition and biological activities of *Isochrysis galbana* T-ISO, *Tetraselmis* sp. and *Scenedesmus* sp.: possible application in the pharmaceutical and functional food industries, *J. Appl. Phycol.* 26 (2014) 151–161.
- [3] E. Ryckeboesch, C. Bruneel, R. Termote-Verhalle, K. Gooris, K. Muylaert, I. Foubert, Nutritional evaluation of microalgae oils rich in omega-3 long chain

- polyunsaturated fatty acids as an alternative for fish oil, *Food Chem.* 160 (2014) 393–400.
- [4] E. Tibaldi, G. Chini Zittelli, G. Parisi, M. Bruno, G. Giorgi, F. Tulli, S. Venturini, M. Tredici, B. Poli, Growth performance and quality traits of European sea bass (*D. labrax*) fed diets including increasing levels of freeze-dried *Isochrysis* sp.(T-ISO) biomass as a source of protein and n-3 long chain PUFA in partial substitution of fish derivatives, *Aquaculture* 440 (2015) 60–68.
- [5] S.M. Kim, S.W. Kang, O.N. Kwon, D. Chung, C.H. Pan, Fucoxanthin as a major carotenoid in *Isochrysis* aff. *galbana*: Characterization of extraction for commercial application, *J. Korean Soc. Appl. Biol. Chem.* 55 (2012) 477–483.
- [6] A. Niccolai, E. Bigagli, N. Biondi, L. Rodolfi, L. Cinci, C. Luceri, M.R. Tredici, In vitro toxicity of microalgal and cyanobacterial strains of interest as food source, *J. Appl. Phycol.* 29 (2017) 199–209.
- [7] T.M. Mata, A.A. Martins, N.S. Caetano, Microalgae for biodiesel production and other applications: a review, *Renew. Sust. Energ. Rev.* 14 (2010) 217–232.
- [8] C. Herrero, J. Abalde, J. Fabregas, Nutritional properties of four marine microalgae for albino rats, *J. Appl. Phycol.* 5 (1993) 573–580.
- [9] K. Nuno, A. Villarruel-Lopez, A.M. Puebla-Perez, E. Romero-Velarde, A.G. Puebla-Mora, F. Ascencio, Effects of the marine microalgae *Isochrysis galbana* and *Nannochloropsis oculata* in diabetic rats, *J. Funct. Foods* 5 (2013) 106–115.
- [10] A.B. Nair, S. Jacob, A simple practice guide for dose conversion between animals and human, *J. Basic Clin. Pharm.* 7 (2) (2016) 27–31.
- [11] M.R. Tredici, N. Bassi, M. Prussi, N. Biondi, L. Rodolfi, G. Chini Zittelli, G. Sampietro, Energy balance of algae biomass production in a 1-ha "Green Wall Panel" plant: how to produce algae biomass in a closed reactor achieving a high Net Energy Ratio, *Appl. Energy* 154 (2015) 1103–1111.
- [12] M. Dubois, K.A. Gilles, J.K. Hamilton, P. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, *Anal. Chem.* 28 (3) (1956) 350–356.
- [13] J.B. Marsh, D.B. Weinstein, Simple charring method for determination of lipids, *J. Lipid Res.* 7 (1966) 574–576.
- [14] C. Castagnini, C. Luceri, S. Toti, E. Bigagli, G. Caderni, A.P. Femia, L. Giovannelli, M. Lodovici, V. Pitozzi, M. Salvadori, L. Messerini, R. Martin, E.G. Zoetendal, S. Gaj, L. Eijssen, C.T. Evelo, C.M. Renard, A. Baron, P. Dolara, Reduction of colonic inflammation in HLA-B27 transgenic rats by feeding Marie Ménard apples. Rich in polyphenols, *Br. J. Nutr.* 102 (2009) 1620–1628.
- [15] A.P. Femia, C. Luceri, S. Toti, A. Giannini, P. Dolara, G. Caderni, Gene expression profile and genomic alterations in colonic tumours induced by 1,2-dimethylhydrazine (DMH) in rats, *BMC Cancer* 10 (2010), <https://doi.org/10.1186/1471-2407-10-194>.
- [16] V. Mimouni, L. Ulmann, V. Pasquet, M. Mathieu, L. Picot, G. Bougaran, J.-P. Cadoret, A. Morant-Manceau, B. Schoefs, The potential of microalgae for the production of bioactive molecules of pharmaceutical interest, *Curr. Pharm. Biotechnol.* 13 (2012) 2733–2750.
- [17] Z. Zhu, S. Zhu, Z. Wu, D. Liu, Y. Yang, X. Wang, J. Zhu, M. Tepel, Effect of sodium on blood pressure, cardiac hypertrophy, and angiotensin receptor expression in rats, *Am. J. Hypertens.* 17 (2004) 21–24.
- [18] G. Gutiérrez-Salmeán, L. Fabila-Castillo, G. Chamorro-Cevallos, Nutritional and toxicological aspects of *Spirulina* (*Arthrospira*), *Nutr. Hosp.* 32 (2015) 34–40.
- [19] E. Bigagli, L. Cinci, A. Niccolai, M. Tredici, N. Biondi, L. Rodolfi, M. Lodovici, M. D'Ambrosio, G. Mori, C. Luceri, Safety evaluations and lipid-lowering activity of an *Arthrospira platensis* enriched diet: a 1-month study in rats, *Food Res. Int.* 102 (2017) 380–386.
- [20] R. Bluestone, S. Brady, J. Waisman, J.R. Klinenberg, Experimental hyperuricemic nephropathy. A model for human urate deposition disease, *Arthritis Rheum.* 18 (1975) 823–834.
- [21] Y. Park, W.S. Harris, Dose-response of n-3 polyunsaturated fatty acids on lipid profile and tolerability in mildly hypertriglyceridemic subjects, *J. Med. Food* 12 (2009) 803–808.
- [22] L. Yang, Z. Song, W. Cao, Y. Wang, H. Lu, F. Guo, H. Yang, J. Chen, S. Wang, G. Sun, Effects of diets with different n-6/n-3 fatty acids on cardiovascular risk factors in mice fed high-fat diets, *Wei Sheng Yan Jiu* 45 (2016) 436–441.
- [23] M.N. Woo, S.M. Jeon, H.J. Kim, M.K. Lee, S.K. Shin, Y.C. Shin, Y.B. Park, M.S. Choi, Fucoxanthin supplementation improves plasma and hepatic lipid metabolism and blood glucose concentration in high-fat fed C57BL/6N mice, *Chem. Biol. Interact.* 186 (2010) 316–322.
- [24] T. Hashimoto, Y. Ozaki, M. Taminato, S.K. Das, M. Mizuno, K. Yoshimura, T. Maoka, K. Kanazawa, The distribution and accumulation of fucoxanthin and its metabolites after oral administration in mice, *Br. J. Nutr.* 102 (2009) 242–248.
- [25] O.A. Gudbrandsen, H. Wergedahl, B. Liaset, M. Espe, R.K. Berge, Dietary proteins with high isoflavone content or low methionine-glycine and lysine-arginine ratios are hypocholesterolaemic and lower the plasma homocysteine level in male Zucker fa/fa rats, *Br. J. Nutr.* 94 (2005) 321–323.
- [26] M.R. Brown, C.D. Garland, S.W. Jeffrey, I.D. Jameson, J.M. Leroi, The gross and amino acid compositions of batch and semi-continuous cultures of *Isochrysis* sp. (clone T.ISO), *Pavlova lutheri* and *Nannochloropsis oculata*, *J. Appl. Phycol.* 5 (1993) 285–296.
- [27] C. Lavigne, A. Marette, H. Jacques, Cod and soy proteins compared with casein improve glucose tolerance and insulin sensitivity in rats, *Am. J. Physiol. Endocrinol. Metab.* 278 (2000) 491–500.
- [28] A. Grasa-López, Á. Miliar-García, L. Quevedo-Corona, N. Paniagua-Castro, G. Escalona-Cardoso, E. Reyes-Maldonado, M.E. Jaramillo-Flores, *Undaria pinnatifida* and fucoxanthin ameliorate lipogenesis and markers of both inflammation and cardiovascular dysfunction in an animal model of diet-induced obesity, *Mar. Drugs* 14 (2016) E148, <https://doi.org/10.3390/md14080148>.
- [29] H. Maeda, M. Hosokawa, T. Sashima, K. Funayama, K. Miyashita, Fucoxanthin from edible seaweed, *Undaria pinnatifida*, shows antiobesity effect through UCP1 expression in white adipose tissues, *Biochem. Biophys. Res. Commun.* 332 (2005) 392–397.
- [30] Y. Ishigaki, H. Katagiri, T. Yamada, T. Ogihara, J. Imai, K. Uno, Y. Hasegawa, J. Gao, H. Ishihara, T. Shimosegawa, H. Sakoda, T. Asano, Y. Oka, Dissipating excess energy stored in the liver is a potential treatment strategy for diabetes associated with obesity, *Diabetes* 54 (2005) 322–332.
- [31] J.K. Kim, J.J. Fillmore, Y. Chen, C. Yu, I.K. Moore, M. Pypaert, E.P. Lutz, Y. Kako, W. Velez-Carrasco, I.J. Goldberg, J.L. Breslow, G.I. Shulman, Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001) 7522–7527.