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

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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 PPARγ ($p < 0.05$) 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 ω3) [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 ω3) 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 × 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 NanoPhotometer 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-carotenal (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 × 250 mm, 5 μm particle size) (Waters, Massachusetts, USA), and a UV photodioide 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 μg mL-1 in methanol/MTBE 4:1), with β-apo-carotenal (50 μg/mL) and Sudan Red (90 μg/mL) 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 (μg/mL) 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 | *T. lutea* 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.