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13-week dietary study and *in vitro* and *in vivo* genotoxicity studies of a structuring fat produced through a microalgal fermentation process

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ABSTRACT

Microalgae are increasingly being utilized as food ingredients for a variety of applications, including as sources of protein, egg and dairy substitutes, and cooking oils. The dietary safety of a new structuring fat produced using a heterotrophic fermentation process by a strain of *Prototheca moriformis* was evaluated in a 13-week dietary toxicity study and compared with kokum fat, a structuring fat of similar composition used in the food industry and derived from *Garcinia indica* seeds. The algal structuring fat was evaluated for its genotoxic potential using both *in vitro* and *in vivo* assays. No treatment-related adverse events occurred in rats consuming algal structuring fat or kokum fat in the 13-week study; no treatment-related effects were reported for body weight, food consumption, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, or histopathology. While statistically significant effects occurred in some parameters, none were dose-related or considered adverse. Overall, the NOAELs for the algal structuring fat and the kokum fat were 100 000 ppm, the highest concentrations tested. The algal structuring fat was not mutagenic in the bacterial reverse mutation assay in the *Salmonella typhimurium* or *Escherichia coli* strains tested and was not clastogenic in the *in vivo* mouse bone marrow chromosome aberration assay.

1. Introduction

There are very few natural fats that have the unique melting properties of cocoa butter. The sharp melting profile associated with cocoa butter, which approximates human body temperature, yet its ability to remain solid at room temperature, derives from its high concentration of structuring fats (*i.e.*, symmetrical monounsaturated triglycerides in which oleate (C18:1) occupies the sn-2 position) in greater than 80% of the triacylglycerol (TAG) species present [18]. Kokum butter has been evaluated for supplementation to cocoa butter, but cost still prohibits widespread use of this fat [13]. While fractionation and hydrogenation processes have been developed to increase levels of structuring fats in vegetable and lauric acid-containing (palm kernel) oils and thereby impart melt profiles similar to cocoa butter, their costs of production are relatively high, their uses limited because of the type of TAG species found in the starting oil [4], and there are negative health consequences associated with the generation of trans fats when hydrogenation is used to make cocoa butter-like fats. Trans fats have been found to increase low-density lipoprotein (LDL) levels, increasing the risk of cardiovascular disease [9,15]. Indeed, the food industry is moving away from the use of trans fats and partially hydrogenated oils due to an increased understanding of the potential adverse effects related to their consumption, and the US FDA's decision to rescind the Generally Recognized As Safe (GRAS) status of partially hydrogenated oils (PHOs) [6]. Hence, the search to find replacements and meet additional demand for structuring fats will only become more challenging.

New sources of oils that can either replace or complement currently available oil sources includes the use of microalgae that have been found to produce high levels of potentially useful oils [19]. Advances in production and processing have made

Abbreviations: 2-AA, 2-aminoanthracene; AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care International; ANOVA, Analysis of Variance; AOAC, Association of Analytical Communities; AOCS, American Oil Chemists' Society; ASTM, American Society for Testing and Materials; bw, Body weight; GLP, Good laboratory practice; CPA, Cyclophosphamide; cps, Centipoise; DHA, Docosahexaenoic acid; g, Gram; EPA, Eicosapentaenoic acid; GRAS, Generally recognized as safe; GRN, GRAS notification; ISO, International Organization for Standardization; kg, Kilogram; LDL, Low-density lipoprotein; mg, Milligram; MMS, Methylmethansulfonate; MTD, Maximum tolerated dose; ppm, Parts-per-million; 4-NOPD, 4-nitro-*o*-phenylene-diamine; NOAEL, No-observed-adverse-effect level; OECD, Organisation for Economic Cooperation and Development; OSD, Open source diet; PHOs, Partially hydrogenated oils; RSD, Relative standard deviation; SOS, Stearic-oleic-stearic triglyceride; TAG, Triacylglycerol; TFA, Total fatty acid; US FDA, United States Food and Drug Administration.

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the manufacture of new microalgal-derived oils and ingredients through heterotrophic fermentation more cost-effective. The ability to genetically engineer certain species of microalgae, as well as tightly controlling their growing conditions, has resulted in the production of oils with well-defined fatty-acid constituents possessing desirable properties.

Prototheca moriformis is an achlorophyllous (*i.e.*, non-chlorophyll producing) microalga related to *Chlorella protothecoides* (aka *Auxenochlorella protothecoides*) and is found ubiquitously in the environment [16]. A strain of *P. moriformis* was genetically engineered as a stable microorganism that produces significant amounts of a new structuring fat containing mostly stearic (~55%) and oleic (~35%) fatty acids, with minor amounts of other fatty acids. The resulting fat is composed primarily of triglycerides (>98%), with minor levels of diglycerides and monoglycerides (<2%). This structuring fat produced by this engineered organism has been produced for use in a variety of food products, but has not previously been added to food. The algal structuring fat is similar to kokum butter, a fat utilized in the European Union (EU) as a substitute for cocoa butter [5].

The typical fatty acid profile for kokum butter is: palmitic acid (2–6%), stearic acid (50–62%), linoleic (0–2%) and oleic acid (30–42%) [1]. Before introducing a new food ingredient to the market for human consumption, a demonstration of the safety of that ingredient must be completed.

To evaluate the dietary safety of the genetically engineered microalgal-derived structuring fat produced using a heterotrophic fermentation process, the structuring fat was assessed for toxicity in a comprehensive 13-week dietary study in rats and compared to another structuring fat containing similar levels of stearate (kokum fat derived from the seeds of *Garcinia indica*). No safety information could be located in the scientific literature evaluating the safety of kokum fat according to current scientific standards, and this is also the first scientific evaluation of the structuring fat produced by this genetically engineered strain of *P. moriformis*. The comparison was particularly relevant as the algal structuring fat has a fatty acid and TAG composition quite similar to kokum [12], yet the latter is already consumed in the European Union [5] and in India [7]. An *in vitro* mutagenicity study in bacteria (the bacterial reverse mutation study) and the *in vivo* chromosome aberration assay were also conducted on the algal structuring fat to evaluate its clastogenic potential.

2. Materials and methods

2.1. Test substance and diet preparation

The algal structuring fat (lot # RBD735) is an off-white, refined, bleached, deodorized solid, isolated from a genetically engineered strain of *P. moriformis* utilizing a unique manufacturing process to produce the structuring fat with consistent fatty acid ratios. The neutral oil is composed of >95% triglycerides, followed by diglycerides (<2%) and monoglycerides (<0.5%). The major fatty acids are stearic acid (~55%) and oleic acid (~35%), as reported as the area percent of total fatty acids. Nonsaponifiable material is less than 1% and the moisture content is approximately 113 ppm (0.01%). Product characteristics of the algal structuring fat are provided in Table 1.

A comprehensive screen for toxins was carried out on the oil. Pheophorbide A is a naturally-occurring degradation product of chlorophyll that is associated with photosensitive dermatitis [10]. Although this organism is achlorophyllous, the test fat was analyzed for pheophorbide A by high-performance liquid chromatography with fluorescence detection at UBE Analytical Laboratories (Fullerton, CA).[1] The fat was also analyzed for the following algal and cyanotoxins: amnesic shellfish poisoning toxins (domoic acid), diarrhetic shellfish poisoning toxins (okadaic acid, dinophysistoxin-1, pectenotoxin-2, azaspiracid-1, yessotoxin, and homo-yessotoxin), paralytic shellfish poisoning toxins (gonyautoxins 1–6; decarbamoylgonyautoxins 2 and 3; saxitoxin, decarbamoylsaxitoxin, neosaxitoxin and ciguatoxins 1–4), cyanobacterial toxins (microcystin-RR, -YR, -LR, -LW, -LF, -LA, -WR, -LY and -HtyR and dm-microcystin-RR and -LR), nodularin, anatoxin and cylindrospermopsin. The algal and cyanotoxin assays were conducted by liquid chromatography with tandem mass spectrometric detection[2] at the Food GmbH Jena Analytik - Consulting (Jena, Germany). No toxins were reported above detection limits (data not shown).

Diets were formulated using the supplied basal Open Standard Diet obtained from Research Diets, Inc. (New Brunswick, NJ) to which kokum and the algal structuring fat were added to achieve the target doses (with soybean oil as the balance) and to provide comparable fat, protein and carbohydrate content across dose groups. All test and control diets were prepared approximately weekly and stored under refrigeration until used. The concentration of test substance and the reference kokum, stability and homogeneity were evaluated via analysis of the fats in collected feed samples (samples frozen until assayed).

2.2. Chemicals and materials

Corn oil and cyclophosphamide (CPA) were purchased from Sigma–Aldrich Corp. (St. Louis, MO) for the chromosomal aberration assay. Kokum and algal structuring fats were provided by Solazyme, Inc. (South San Francisco, CA) for the 13-week subchronic dietary study. The kokum fat was obtained from Essential Wholesale & Labs (Portland, OR). The S9 metabolic activation mix for the bacterial reverse mutation assay was purchased from Molecular Toxicology, Inc. (Boone, NC) where it was prepared from livers of male Sprague-Dawley rats induced with phenobarbital and benzoflavone. The bacterial reverse mutation assay positive control substances, sodium azide (NaN_3), ICR 191 acridine, daunomycin, methylmethanesulfonate (MMS) and 2-aminoanthracene (2-AA), were also purchased from Molecular Toxicology, Inc. (Boone, NC), as well as the overlay agar (supplemented with biotin and limited amounts of histidine and tryptophan) and minimal glucose agar plates.

2.3. Animals and organisms

CRL Sprague-Dawley (SD) CD® IGS rats (male and female) were obtained from Charles River Laboratories (Raleigh, NC) for the 13-week subchronic dietary study. Veterinarian staff visually inspected all rats at delivery and during a five-day acclimation phase prior to study initiation. The rats were 7–8 weeks of age at study initiation. Body weight variations remained within ±20% of the measured mean for both sexes at study start (220.7 g mean for the males with a range of 220.3–221.5 g and a 182.0 g mean for the females with a range of 181.7–182.3 g). The rats were individually housed in suspended stainless steel caging in a temperature (19–23 °C) and humidity (32–55%) controlled room with a 12-h light/dark cycle. Filtered water and the test diet formulations were provided *ad libitum*.

The bacterial strains (*Salmonella typhimurium* TA1535, TA1537, TA98, TA 100 and *Escherichia coli WP2 uvrA) utilized for the bacterial reverse mutation assays performed at Product Safety Laboratories*

[1] Limit of detection was 0.5 ppm for pheophorbide A.
[2] Limits of detection ranged from 0.0008 to 0.1 μg/g in the fat.

Table 1
Product characteristics for algal structuring fat test material.

Parameter (method)	Assay result	Parameter (method)	Assay result
Compositional properties			
Insoluble impurities (AOCS Ca 3a-46)	<0.01%	Iodine value (internal method)	35.2
Free fatty acids, oleic (AOCS Ca 5a-40)	0.47%	Peroxide value (AOCS Cd 8–53)	0 meq/kg
Acid value (AOCS Ca 5a-40)	0.94 mg KOH/g	*p*-Anisidine value (ISO 6885)	0.3
Neutral oil (AOCS Ca 9f-57)	99.2%	Oxidized triglycerides (IUPAC 2.507 and 2.508)	0.55% (polar basis)
Unsaponifiable matter (AOCS Ca 6a-40)	0.63%	Polymerized triglycerides (IUPAC 2.507 and 2.508)	0.18% (polar basis)
Triglycerides (AOCS Cd 11d-96)	98.7%	Residual hexane (internal method)	<1.0
Monoglycerides (AOCS	<0.01%	Elemental analysis (AOCS Ca20–99 and Ca 17–01)	
Cd 11d-96)		Mercury	<0.20 ppm
Diglycerides (AOCS Cd	1.8%	Cadmium	<0.03 ppm
11d-96)		Chromium	<0.02 ppm
Total polar compounds	3.7%	Lead	<0.20 ppm
(AOCS Cd 20–91)		Arsenic	<0.20 ppm
Fatty acids (C-M-00036-000)			
C16:0 (Palmitic)	4.2 area%	TAG profile	
C18:0 (Stearic)	55.1 area%	Saturate–saturate–saturate	<5%
C18:1 (Oleic)	35.0 area%	Saturate–unsaturate–saturate	>75%
Total FAMEs identified	98.8 area%	Saturate–oleic–saturate	>70%

AOCS: American Oil Chemists' Society; ASTM: American Society for Testing and Materials; FAME: Fatty Acid Methyl Ester; ISO: International Organization for Standardization; IUPAC: International Union of Pure and Applied Chemistry; OSI: Oil Stability Index.

(PSL; Dayton, NJ) were obtained from Molecular Toxicology, Inc. (Boone, NC).

Eurofins BioPharma Product Testing Munich GmbH (Planegg, Germany) conducted the chromosomal aberration assay using male and female NMRI mice from Charles River GmbH (Sulzfield, Germany) for the *in vivo* segment. The mice were visually inspected at delivery and during the acclimation period (minimum of five days) by in-house veterinary staff. The mice were a minimum of seven weeks old at the time of study initiation. The initial body weights ranged from 31.4 to 38.2 g for the males (9.9% bw variation) and 26.2–31.6 g for the female mice (9.6% bw variation). The mice were housed by sex (five animals/cage) in animal rooms environmentally controlled ($22 \pm 3\,^{\circ}C$ and $55 \pm 10\%$ relative humidity) and on a twelve-hour photoperiod. Tap water (sulfur acidified to ~pH 2.8) and Altromin 1324 maintenance diet (Altromin Spezialfutter GmbH & Co.) were provided *ad libitum*.

2.4. Guidelines

The 13-week rat dietary study design conformed to the following guidelines:

- Organisation for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Test No. 408): Health Effects, Repeated Dose 90-Day Oral Toxicity Study in Rodents (1998).
- U.S. FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C.4.a. (2007).

The bacterial reverse mutation assay was performed in conformance with:

- OECD Guidelines for Testing of Chemicals, Section 4 (Test No. 471): "Bacterial Reverse Mutation Test", adopted July 21st, 1997.
- U.S. FDA Redbook. I.V.C.1.a. Short-Term Tests for Genetic Toxicity (2000): Bacterial Reverse Mutation Test.

The mammalian bone marrow chromosome aberration test was conducted to comply with:

- OECD Guidelines for Testing of Chemicals, Section 4, No. 475, "Mammalian Bone Marrow Chromosome Aberration Test", adopted July 21st, 1997.
- Commission Regulation (EC) No. 440/2008 B.11: "Mutagenicity—Mammalian Bone Marrow Chromosome Aberration Test", dated May 30, 2008.
- EPA Health Effects Test Guidelines, OPPTS 870.5385 "Mammalian Bone Marrow Chromosome Aberration Test", EPA 712-C-98-225, August 1998.
- First Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 420, "Acute Oral Toxicity—Fixed Dose Procedure" Adopted December 17, 1998.
- OECD Guidelines for Testing of Chemicals, Section 4, No. 423, "Acute Oral Toxicity—Acute Toxic Class Method" adopted December 17, 2001.

2.5. Experimental design

2.5.1. Thirteen-week dietary study

The dietary study used to evaluate the potential of the algal structuring fat or kokum fat to produce toxicity followed well-established, internationally-accepted protocol guidelines, which have been utilized to evaluate the potential for toxicological effects of other algal-based food ingredients [20–22]. Sprague-Dawley rats (CD® IGS) were randomized to receive a basal control diet (Group 1, placebo control; $n = 20$/sex) or one of six test diets (n = ten/sex/group) formulated to contain either kokum fat at 25 000 ppm (Group 2), 50 000 ppm (Group 3) and 100 000 ppm (Group 4), or dietary levels of algal structuring fat at 25 000 ppm (Group 5), 50 000 ppm (Group 6) and 100 000 ppm (Group 7). The algal structuring fat or kokum fat replaced the soybean oil content of the basal control diet, such that the test and control diets had equivalent fat content. The test and control diets were provided to the rats *ad libitum* throughout the greater than 90-day study. Following the treatment period, all surviving rats (fasted $\geq$ 15 h) were terminated (by exsanguination) on Days 92/93 (males) or Days 94/95 (females).

During the study, mortality was checked twice daily and viability, signs of gross toxicity and behavioral changes were observed once per day during the study. The rats were observed for a battery of detailed clinical endpoints weekly. Body weights were recorded twice during acclimation, including just prior to test initiation (Day 0), weekly thereafter, on Days 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 83, 91 and just prior to terminal sacrifice. Because consumption of oils and fats of this type are not thought to alter neurological functions, a specific functional observational batter/motor activity

Table 2
Hematology, coagulation, and clinical chemistry parameters in male rats following dietary treatment with kokum fat or algal structuring fat.

Parameter	Group 1[b] 0 ppm (n = 19)	Group 2 25,000 ppm KF (n = 10)	Group 3 50,000 ppm KF (n = 10)	Group 4 100,000 ppm KF (n = 10)	Group 5 25,000 ppm AF (n = 10)	Group 650,000 ppm AF (n = 10)	Group 7 100,000 ppm AF (n = 10)
Hematology—week 12							
RBC (10^6/μl)	8.63 ± 0.29	8.61 ± 0.35	8.53 ± 0.33	8.65 ± 0.23	8.55 ± 0.35	8.67 ± 0.36	8.69 ± 0.32
Hemoglobin (g/dl)	15.5 ± 0.5	15.3 ± 0.4	15.4 ± 0.4	15.4 ± 0.5	15.6 ± 0.5	15.3 ± 0.5	15.7 ± 0.5
Hematocrit (%)	45.6 ± 1.4	45.3 ± 1.1	45.7 ± 1.3	45.9 ± 1.4	45.7 ± 1.8	45.4 ± 1.2	46.4 ± 1.8
MCV (fl)	52.8 ± 1.2	52.8 ± 2.1	53.6 ± 2.5	53.0 ± 1.7	53.5 ± 1.3	52.3 ± 1.2	53.4 ± 0.7
MCH (pg)	17.9 ± 0.5	17.9 ± 0.8	18.1 ± 0.8	17.9 ± 0.6	18.2 ± 0.6	17.6 ± 0.5	18.1 ± 0.2
MCHC (g/dl)	34.0 ± 0.5	33.8 ± 0.4	33.7 ± 0.6	33.7 ± 0.5	34.0 ± 0.6	33.7 ± 0.5	33.8 ± 0.4
RDW (%)	12.7 ± 0.5	12.6 ± 0.7	12.3 ± 0.4	12.2 ± 0.5	12.4 ± 0.5	12.5 ± 0.4	12.3 ± 0.3
Platelet count (10^3/μl)	991 ± 111	999 ± 131	951 ± 182	1086 ± 65	1020 ± 86	1080 ± 119	1077 ± 96
WBC (10^3/μl)	12.14 ± 2.47	11.55 ± 1.60	11.36 ± 2.72	11.93 ± 1.40	10.96 ± 2.17	12.21 ± 2.43	11.74 ± 1.92
ARET (10^3/μl)	184.3 ± 27.1	166.6 ± 15.7	167.8 ± 27.9	163.9 ± 33.4	170.6 ± 12.5	167.1 ± 29.1	161.3 ± 19.7
ANEU (10^3/μl)	2.72 ± 1.38	2.19 ± 0.73	2.21 ± 0.62	2.06 ± 0.62	2.13 ± 0.74	2.53 ± 0.96	2.07 ± 0.68
ALYM (10^3/μl)	8.74 ± 1.66	8.69 ± 1.20	8.47 ± 2.13	9.19 ± 1.29	8.19 ± 1.92	8.93 ± 2.12	8.92 ± 1.39
AMON (10^3/μl)	0.39 ± 0.20	0.35 ± 0.14	0.34 ± 0.10	0.39 ± 0.12	0.35 ± 0.10	0.41 ± 0.09	0.39 ± 0.13
AEOS (10^3/μl)	0.16 ± 0.09	0.17 ± 0.06	0.21 ± 0.10	0.16 ± 0.06	0.17 ± 0.07	0.20 ± 0.06	0.21 ± 0.10
ABAS (10^3/μl)	0.06 ± 0.03	0.06 ± 0.03	0.05 ± 0.03	0.05 ± 0.01	0.05 ± 0.02	0.06 ± 0.02	0.07 ± 0.03
ALUC (10^3/μl)	0.07 ± 0.04	0.08 ± 0.02	0.07 ± 0.04	0.08 ± 0.04	0.08 ± 0.03	0.08 ± 0.03	0.08 ± 0.03
Coagulation—week 13							
PT (sec)	10.9 ± 0.3	10.8 ± 0.3	11.0 ± 0.3	10.8 ± 0.3	10.9 ± 0.3	10.9 ± 0.2	11.0 ± 0.4
APTT (sec)	18.4 ± 2.0	18.7 ± 1.5	21.0 ± 6.2	20.1 ± 2.4	18.2 ± 1.8	19.0 ± 1.8	20.8 ± 4.7
Clinical chemistry—week 12							
AST (U/l)	117 ± 111	98 ± 22	77 ± 9	79 ± 21	91 ± 48	75 ± 11	80 ± 12
ALT (U/l)	47 ± 62	29 ± 12	25 ± 4	32 ± 23	36 ± 26	25 ± 4	27 ± 5
SDH (U/l)	9.8 ± 10.1	6.2 ± 5.8	6.3 ± 1.5	9.1 ± 4.8	8.0 ± 4.8	6.6 ± 1.0	5.8 ± 1.7
ALKP (U/l)	95 ± 29	82 ± 24	86 ± 22	93 ± 30	125 ± 55	90 ± 24	102 ± 36
BILI (mg/dl)	0.14 ± 0.02	0.14 ± 0.03	0.14 ± 0.01	0.14 ± 0.2	0.15 ± 0.02	0.15 ± 0.02	0.15 ± 0.03
BUN (mg/dl)	12 ± 1 A	11 ± 1	10 ± 1	12 ± 1	11 ± 1	11 ± 1	10 ± 1*
Creatinine (mg/dl)	0.27 ± 0.03	0.29 ± 0.03	0.27 ± 0.03	0.27 ± 0.02	0.27 ± 0.03	0.28 ± 0.04	0.25 ± 0.03
Total cholesterol (mg/dl)	82 ± 27	87 ± 26	83 ± 20	93 ± 15	83 ± 14	80 ± 17	81 ± 16
Triglycerides (mg/dl)	80 ± 34	72 ± 33	87 ± 46	91 ± 31	88 ± 26	78 ± 25	96 ± 34
Glucose, fasting (mg/dl)	122 ± 15	128 ± 12	131 ± 17	115 ± 20	118 ± 15	116 ± 17	122 ± 21
Total protein (g/dl)	6.5 ± 0.2	6.6 ± 0.4	6.5 ± 0.2	6.5 ± 0.2	6.6 ± 0.3	6.5 ± 0.3	6.3 ± 0.4
Albumin (g/dl)	3.1 ± 0.2	3.2 ± 0.2	3.2 ± 0.2	3.1 ± 0.2	3.1 ± 0.2	3.2 ± 0.2	3.0 ± 0.2
Globulin (g/dl)	3.4 ± 0.1	3.4 ± 0.3	3.3 ± 0.1	3.3 ± 0.2	3.5 ± 0.2	3.3 ± 0.2	3.3 ± 0.2
Calcium (mg/dl)	10.1 ± 0.3	9.8 ± 0.4	10.1 ± 0.3	10.2 ± 0.2	10.3 ± 0.4	10.2 ± 0.4	10.1 ± 0.3
Inorganic phosphorus (mg/dl)	6.2 ± 0.5	6.3 ± 0.6	6.1 ± 0.5	6.4 ± 0.5	6.6 ± 0.5	6.4 ± 0.5	6.6 ± 0.3
Sodium (mmol/l)	145.5 ± 7.6	149.9 ± 10.1	142.7 ± 9.0	144.3 ± 9.6	147.1 ± 10.0	142.6 ± 7.3	140.0 ± 4.5
Potassium (mmol/l)	5.01 ± 0.54	5.51 ± 0.86	4.92 ± 0.37	4.72 ± 0.41	5.17 ± 0.48	4.92 ± 0.46	4.89 ± 0.35
Chloride (mmol/l)	104.9 ± 5.4	108.4 ± 7.6	103.5 ± 6.4	104.2 ± 7.1	106.2 ± 7.5	103.9 ± 5.2	102.2 ± 3.8
–							
Urinalysis—week 12							
Urine volume (ml)	6.8 ± 4.4	3.2 ± 2.6	6.6 ± 2.7	4.3 ± 2.2	4.9 ± 5.6	5.9 ± 3.7	5.9 ± 4.6
pH	6.4 ± 0.5	6.2 ± 0.4[a]	6.3 ± 0.3	6.2 ± 0.2	6.2 ± 0.3	6.5 ± 0.4	6.1 ± 0.2
Specific gravity	1.048 ± 0.022	1.069 ± 0.022[a]	1.046 ± 0.017	1.063 ± 0.019	1.062 ± 0.027	1.048 ± 0.022	1.048 ± 0.018
URO (EU/dl)	0.3 ± 0.3	0.3 ± 0.3[a]	0.3 ± 0.3	0.2 ± 0.0	0.4 ± 0.4	0.3 ± 0.3	0.3 ± 0.3
UMTP (mg/dl)	139 ± 74	297 ± 181	183 ± 106	237 ± 104[a]	167 ± 77[a]	155 ± 90	157 ± 63

ABAS, absolute basophils; AEOS, absolute eosinophils; AF = algalfat; ALKP, alkaline phosphatase; ALT, alanine aminotransferase; ALUC, absolute large unstained cells; ALYM, absolute lymphocytes; AMON, absolute monocytes; ANEU, absolute neutrophils; APTT, activated partial thromboplastin time; ARET, absolute reticulocytes; AST, aspartate aminotransferase; BILI, total bilirubin; BUN, blood urea nitrogen; KF = kokum fat; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PT, prothrombin time; RBC, erythrocytes; RDW, red cell distribution width; SDH, sorbitol dehydrogenase; UMTP, protein; URO, urobilinogen; WBC, total white blood cells.
A = Significantanalysis of variance.
* Statistically significant from Group 1 control (P < 0.05).
[a] n = 9.
[b] Control group.

assessment was not conducted. However, a detailed observation was conducted on a weekly basis that included evaluation for changes in gait, posture, and response to handling, as well as the presence of clonic or tonic movements (*e.g.*, excessive grooming or repetitive behavior) or bizarre behavior (*e.g.*, self-mutilation, walking backwards) were recorded. Individual food consumption was also recorded weekly and just prior to terminal sacrifice on the same schedule as body weight measurements. During the acclimation period, the eyes of all rats being considered for the study were examined by focal illumination, indirect ophthalmoscopy and, when indicated, slit-lamp microscopy. Mydriatic eye drops were administered prior to ophthalmoscopy and the eyes were examined in subdued light; these procedures were repeated on all surviving animals on Day 88 of the study. Urine samples were collected on Day 86 and Day 88 for males and females, respectively, from all surviving animals. The urine was examined for quality, color, clarity, volume, pH, glucose, specific gravity, protein, ketone, bilirubin, blood, urobilinogen and microscopic sediments. Blood was sampled on Day 86 and Day 88 for all surviving males and females, respectively, for hematology and clinical chemistry analysis, and on Day 92/93 (males) or Day 94/95 (females) for coagulation assessments for all surviving animals prior to necropsy (Tables 2 and 3). Blood was collected from all animals for hematology and clinical chemistry analysis by sublingual bleeding under isoflurane anesthesia. Blood samples used to determine the prothrombin time and the activated partial thromboplastin time (coagulation)

Table 3
Hematology, coagulation, and clinical chemistry parameters in female rats following dietary treatment with kokum fat or algal structuring fat.

Parameter	Group 1[d] 0 ppm ($n=19$)	Group 2 25,000 ppm KF ($n=10$)	Group 3 50,000 ppm KF ($n=10$)	Group 4 100,000 ppm KF ($n=10$)	Group 5 25,000 ppm AF ($n=10$)	Group 6 50,000 ppm AF ($n=10$)	Group 7 100,000 ppm AF ($n=10$)
Hematology—week 12							
RBC ($10^6/\mu l$)	8.17 ± 0.28	8.11 ± 0.38	7.93 ± 0.33	8.11 ± 0.46	8.20 ± 0.36	8.16 ± 0.33	8.31 ± 0.21
Hemoglobin (g/dl)	15.3 ± 0.5	15.0 ± 0.6	14.6 ± 0.6*	15.1 ± 0.7	15.2 ± 0.5	15.1 ± 0.6	15.4 ± 0.6
Hematocrit (%)	44.5 ± 1.3	43.9 ± 1.7	42.7 ± 1.8	44.2 ± 1.9	44.9 ± 1.5	44.1 ± 1.4	45.3 ± 1.5
MCV (fl)	54.6 ± 1.7	54.2 ± 0.7	53.9 ± 1.0	54.6 ± 1.3	54.7 ± 1.7	54.0 ± 1.0	54.6 ± 1.6
MCH (pg)	18.8 ± 0.6	18.5 ± 0.3	18.4 ± 0.4	18.7 ± 0.6	18.6 ± 0.6	18.5 ± 0.4	18.5 ± 0.5
MCHC (g/dl)	34.4 ± 0.4	34.2 ± 0.4	34.2 ± 0.4	34.2 ± 0.6	33.9 ± 0.6	34.2 ± 0.4	34.0 ± 0.4
RDW (%)	11.5 ± 0.4	11.5 ± 0.5	11.5 ± 0.4	11.5 ± 0.3	11.3 ± 0.5	11.5 ± 0.3	11.4 ± 0.2
Platelet count ($10^3/\mu l$)	978 ± 98	1024 ± 101	1027 ± 121	1117 ± 105*	947 ± 77	1033 ± 135	1042 ± 85
WBC ($10^3/\mu l$)	7.61 ± 2.12	8.14 ± 1.48	7.10 ± 1.57	9.37 ± 2.36	8.02 ± 1.02	8.22 ± 1.58	8.89 ± 2.80
ARET ($10^3/\mu l$)	152.5 ± 34.9	146.5 ± 37.2	148.2 ± 30.2	148.9 ± 20.6	164.6 ± 39.5	136.7 ± 26.7	153.1 ± 28.1
ANEU ($10^3/\mu l$)	1.43 ± 0.90	1.39 ± 0.52	1.28 ± 0.23	1.82 ± 0.92	1.62 ± 0.39	1.30 ± 0.64	1.30 ± 0.56
ALYM ($10^3/\mu l$)	5.76 ± 1.26	6.25 ± 1.18	5.44 ± 1.43	6.91 ± 1.88	5.88 ± 0.74	6.43 ± 1.10	7.05 ± 2.31
AMON ($10^3/\mu l$)	0.23 ± 0.09	0.28 ± 0.08	0.20 ± 0.06	0.34 ± 0.08*	0.26 ± 0.08	0.24 ± 0.09	0.27 ± 0.09
AEOS ($10^3/\mu l$)	0.12 ± 0.05 A	0.13 ± 0.03	0.11 ± 0.02	0.18 ± 0.05*	0.15 ± 0.05	0.14 ± 0.03	0.14 ± 0.05
ABAS ($10^3/\mu l$)	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.02	0.03 ± 0.01
ALUC ($10^3/\mu l$)	0.05 ± 0.02	0.07 ± 0.02	0.05 ± 0.02	0.08 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.09 ± 0.05
Coagulation—week 13							
PT (sec)	10.2 ± 0.2	10.3 ± 0.2	10.3 ± 0.3	10.3 ± 0.3	10.4 ± 0.2	10.4 ± 0.2	10.4 ± 0.2
APTT (sec)	16.7 ± 1.3	16.1 ± 0.9	17.5 ± 2.0	16.7 ± 1.6	16.2 ± 1.5	16.9 ± 1.4	17.7 ± 2.2
Clinical chemistry—week 12							
AST (U/l)	68 ± 17	63 ± 11	69 ± 10	68 ± 28	67 ± 13	63 ± 9[a]	63 ± 9
ALT (U/l)	24 ± 14	18 ± 3	19 ± 2	27 ± 19	21 ± 8	19 ± 4	20 ± 6
SDH (U/l)	6.9 ± 2.7	6.1 ± 1.5	6.5 ± 2.7	8.6 ± 4.4	7.5 ± 3.8	6.2 ± 1.7[a]	5.7 ± 0.9
ALKP (U/l)	47 ± 17	45 ± 15	47 ± 14	52 ± 16	120 ± 210	49 ± 24	40 ± 13
BILI (mg/dl)	0.17 ± 0.03	0.17 ± 0.03	0.17 ± 0.02	0.16 ± 0.04	0.17 ± 0.03	0.17 ± 0.02	0.17 ± 0.02
BUN (mg/dl)	12 ± 2	11 ± 2	11 ± 1	11 ± 1	11 ± 2	11 ± 2	10 ± 2
Creatinine (mg/dl)	0.33 ± 0.05	0.34 ± 0.03	0.34 ± 0.05	0.32 ± 0.02	0.34 ± 0.03	0.33 ± 0.03	0.31 ± 0.04
Cholesterol (mg/dl)	90 ± 27	96 ± 23	89 ± 14	99 ± 25	88 ± 23	90 ± 17	89 ± 19
Triglycerides (mg/dl)	68 ± 41	95 ± 71	71 ± 31	90 ± 55	71 ± 31	81 ± 56	63 ± 20
Glucose, fasting (mg/dl)	105 ± 10	113 ± 14	113 ± 21	112 ± 15	113 ± 16	114 ± 15	115 ± 9
Total protein (g/dl)	7.4 ± 0.5	7.6 ± 0.3	7.4 ± 0.6	7.2 ± 0.7	7.3 ± 0.5	7.4 ± 0.3	7.1 ± 0.4
Albumin (g/dl)	4.0 ± 0.3	4.1 ± 0.2	3.9 ± 0.3	3.8 ± 0.5	4.0 ± 0.3	4.0 ± 0.2	3.8 ± 0.3
Globulin (g/dl)	3.4 ± 0.2	3.5 ± 0.1	3.5 ± 0.3	3.4 ± 0.3	3.4 ± 0.3	3.4 ± 0.2	3.3 ± 0.1
Calcium (mg/dl)	10.6 ± 0.5	10.7 ± 0.2	10.3 ± 0.5	10.6 ± 0.7	10.6 ± 0.5	10.5 ± 0.3	10.5 ± 0.4
Inorganic phosphorus (mg/dl)	5.1 ± 0.9	5.4 ± 0.6	4.4 ± 0.7	5.2 ± 0.6	5.2 ± 0.7	5.1 ± 1.0	5.4 ± 0.5
Sodium (mmol/l)	141.5 ± 3.8	144.0 ± 6.0	142.6 ± 4.4	140.8 ± 4.0	141.8 ± 3.2	141.0 ± 2.6	139.7 ± 1.5
Potassium (mmol/l)	4.22 ± 0.47	4.47 ± 0.38	4.22 ± 0.45	4.42 ± 0.40	4.43 ± 0.40	4.60 ± 0.46	4.42 ± 0.30
Chloride (mmol/l)	101.7 ± 3.1	103.4 ± 4.3	102.9 ± 3.2	101.8 ± 3.0	102.5 ± 2.8	102.1 ± 2.2	101.8 ± 1.1
Urinalysis—week 12							
Urine volume (ml)	4.1 ± 3.2[b]	3.0 ± 2.6	6.3 ± 5.0	6.3 ± 5.7	5.4 ± 4.2	4.1 ± 4.5	5.8 ± 5.2
pH	6.1 ± 0.4[c]	6.1 ± 0.3	6.1 ± 0.2	6.3 ± 0.5	6.3 ± 0.4	6.3 ± 0.5	6.2 ± 0.3[a]
Specific gravity	1.048 ± 0.026[c]	1.061 ± 0.029	1.033 ± 0.014	1.040 ± 0.024	1.039 ± 0.024	1.048 ± 0.019	1.033 ± 0.018[a]
URO (EU/dl)	0.3 ± 0.3[c]	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.3	0.3 ± 0.3	0.2 ± 0.0[a]
UMTP (mg/dl)	98 ± 126[b]	115 ± 73	43 ± 26	107 ± 153	56 ± 43	73 ± 40	80 ± 125

ABAS, absolute basophils; AEOS, absolute eosinophils; AF = algalfat; ALKP, alkaline phosphatase; ALT, alanine aminotransferase; ALUC, absolute large unstained cells; ALYM, absolute lymphocytes; AMON, absolute monocytes; ANEU, absolute neutrophils; APTT, activated partial thromboplastin time; ARET, absolute reticulocytes; AST, aspartate aminotransferase; BILI, total bilirubin; BUN, blood urea nitrogen; KF = kokum fat; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PT, prothrombin time; RBC, erythrocytes; RDW, red cell distribution width; SDH, sorbitol dehydrogenase; UMTP, protein; URO, urobilinogen; WBC, total white blood cells.
A = Significant analysis of variance.
* Statistically significant from Group 1 control ($P < 0.05$).
[a] $n = 9$.
[b] $n = 18$.
[c] $n = 17$.
[d] Control group.

were collected via the inferior vena cava under isoflurane anesthesia at termination. Gross necropsies were performed on all study rats and selected organs and tissues from the control and both high dietary substance groups, as well as organs and tissues of potential toxicologic interest, which were evaluated histologically. Evaluation included examination of the surface of the body, orifices, the thoracic, abdominal and cranial cavities and their contents. The brain, heart, adrenals (combined), kidneys (combined), spleen, liver, thymus, epididymides (combined), ovaries (combined), uterus with oviducts and testes (combined) were isolated and weighed, and organ-to-body weights and organ-to-brain weights were recorded. Organs and tissues (Tables 4 and 5) from all animals were preserved in 10% neutral buffered formalin for possible future histopathological examination. Any organs/tissues that required further examination were trimmed, processed, embedded in paraffin, sectioned with a microtome, placed on glass microscope slides, stained with hematoxylin and eosin and examined by light microscopy. Slide preparation and histopathological assessment were performed by a board-certified veterinary pathologist at Histo-Scientific Research Laboratories (Frederick, MD).

Table 4
Summary of mean terminal body and organ weights in male rats following dietary treatment with kokum fat or algal structuring fat.

Parameter	Group 1[b] 0 ppm (n = 19)	Group 2 25,000 ppm KF (n = 10)	Group 3 50,000 ppm KF (n = 10)	Group 4 100,000 ppm KF (n = 10)	Group 5 25,000 ppm AF (n = 10)	Group 6 50,000 ppm AF (n = 10)	Group 7 100,000 ppm AF (n = 10)
Mean terminal body and organ weights—day92/93							
Terminal body weight (g)	566.2 ± 56.0	574.8 ± 81.2	595.7 ± 54.5	549.6 ± 78.8	562.1 ± 66.4	575.4 ± 57.5	576.6 ± 52.7
Adrenals (g)	0.0604 ± 0.0100 I[1]	0.0636 ± 0.0097	0.0631 ± 0.0091	0.0632 ± 0.0132	0.0559 ± 0.0121	0.0604 ± 0.0081	0.0581 ± 0.0103
Brain (g)	2.277 ± 0.114 I[1]	2.311 ± 0.127	2.289 ± 0.115	2.273 ± 0.116	2.261 ± 0.096	2.281 ± 0.127	2.227 ± 0.086
Epididymides (g)	1.480 ± 0.133 R[2]	1.512 ± 0.204	1.544 ± 0.180	1.576 ± 0.161	1.522 ± 0.249	1.413 ± 0.103	1.441 ± 0.314
Heart (g)	1.647 ± 0.179 I[1]	1.651 ± 0.204	1.619 ± 0.127	1.555 ± 0.189	1.620 ± 0.141	1.614 ± 0.125	1.592 ± 0.124
Kidneys (g)	3.510 ± 0.413 I[1]	3.665 ± 0.612	3.778 ± 0.407	3.450 ± 0.453	3.665 ± 0.363	3.478 ± 0.398	3.490 ± 0.353
Liver (g)	14.102 ± 1.936 I[1]	13.780 ± 3.179	14.245 ± 1.832	13.436 ± 2.169	13.402 ± 2.167	13.037 ± 1.558	12.777 ± 1.567
Spleen (g)	0.889 ± 0.151 I[1]	0.850 ± 0.143	0.885 ± 0.121	0.910 ± 0.129	0.894 ± 0.073	0.857 ± 0.092	0.889 ± 0.118
Testes (g)	3.465 ± 0.269 R[2]	3.563 ± 0.353	3.555 ± 0.279	3.685 ± 0.262	3.526 ± 0.359	3.526 ± 0.341	3.329 ± 0.882
Thymus (g)	0.2987 ± 0.0975 L[3]	0.3350 ± 0.0779	0.3443 ± 0.0850	0.2843 ± 0.0806	0.2689 ± 0.0642	0.2766 ± 0.0437	0.3188 ± 0.0863
Organ-to-body weight ratios[a]							
Adrenals/TBW	0.1081 ± 0.0225 I[1]	0.1118 ± 0.0197	0.1068 ± 0.0185	0.1155 ± 0.0214	0.1003 ± 0.0218	0.1057 ± 0.0164	0.1012 ± 0.0189
Brain/TBW	4.053 ± 0.390 I[1]	4.069 ± 0.394	3.870 ± 0.382	4.194 ± 0.489	4.068 ± 0.463	3.991 ± 0.363	3.892 ± 0.392
Epididymides/TBW	2.6314 ± 0.2868 R[2]	2.6437 ± 0.2915	2.6182 ± 0.4348	2.9178 ± 0.5007	2.7227 ± 0.3946	2.4801 ± 0.3282	2.5245 ± 0.6227
Heart/TBW	2.916 ± 0.266 I[1]	2.880 ± 0.207	2.728 ± 0.199	2.846 ± 0.248	2.895 ± 0.179	2.818 ± 0.215	2.769 ± 0.172
Kidneys/TBW	6.217 ± 0.633 I[1]	6.380 ± 0.597	6.364 ± 0.649	6.321 ± 0.702	6.546 ± 0.431	6.066 ± 0.646	6.098 ± 0.840
Liver/TBW	24.896 ± 2.392 R[2]	23.781 ± 2.499	23.898 ± 2.031	24.555 ± 3.226	23.760 ± 1.659	22.659 ± 1.544**	22.131 ± 1.379***
Spleen/TBW	1.571 ± 0.220 L[3]	1.483 ± 0.180	1.493 ± 0.216	1.677 ± 0.286	1.607 ± 0.206	1.495 ± 0.150	1.540 ± 0.121
Testes/TBW	6.166 ± 0.693 R[2]	6.283 ± 0.898	6.019 ± 0.800	6.801 ± 0.895	6.315 ± 0.702	6.189 ± 0.917	5.838 ± 1.702
Thymus/TBW	0.5280 ± 0.1607 R[2]	0.5872 ± 0.1317	0.5783 ± 0.1439	0.5235 ± 0.1498	0.4781 ± 0.1068	0.4821 ± 0.0707	0.5527 ± 0.1400
Organ-to-brain weight ratios[a]							
Adrenals/BrW	0.0265 ± 0.0044 I[1]	0.0275 ± 0.0038	0.0276 ± 0.0042	0.0277 ± 0.0051	0.0246 ± 0.0047	0.0265 ± 0.0037	0.0262 ± 0.0053
Epididymides/BrW	0.6504 ± 0.0540 R[2]	0.6530 ± 0.0708	0.6735 ± 0.0558	0.6927 ± 0.0537	0.6734 ± 0.1057	0.6218 ± 0.0630	0.6489 ± 0.1447
Heart/BrW	0.724 ± 0.082 I[1]	0.712 ± 0.072	0.707 ± 0.043	0.684 ± 0.070	0.718 ± 0.068	0.709 ± 0.055	0.716 ± 0.063
Kidneys/BrW	1.541 ± 0.160 I[1]	1.579 ± 0.188	1.653 ± 0.186	1.516 ± 0.172	1.621 ± 0.142	1.528 ± 0.181	1.568 ± 0.160
Liver/BrW	6.211 ± 0.950 I[1]	5.918 ± 1.044	6.236 ± 0.861	5.900 ± 0.827	5.930 ± 0.942	5.714 ± 0.569	5.744 ± 0.727
Spleen/BrW	0.391 ± 0.067 I[1]	0.367 ± 0.051	0.387 ± 0.059	0.401 ± 0.056	0.395 ± 0.029	0.376 ± 0.042	0.399 ± 0.051
Testes/BrW	1.523 ± 0.105 R[2]	1.544 ± 0.153	1.556 ± 0.137	1.621 ± 0.070	1.561 ± 0.161	1.552 ± 0.196	1.499 ± 0.399
Thymus/BrW	0.1310 ± 0.0397 L[3]	0.1449 ± 0.0328	0.1496 ± 0.0326	0.1254 ± 0.0364	0.1187 ± 0.0274	0.1210 ± 0.0157	0.1438 ± 0.0416

AF = Algalfat; BrW = Brain weight; KF = Kokum fat; TBW = Terminal body weight.
1[I—Automatic Transformation: Identity (No Transformation)]; 2[R—Automatic Transformation: Rank]; 3[L—Automatic Transformation: Log].
** $P < 0.05$.
*** $P < 0.01$.
[a] Organ-to-body weight ratios and organ-to-brain weight ratio numbers are increased by a factor of 1000 for clarity.
[b] Control group.

2.5.2. Bacterial reverse mutation assays

The potential for the algal structuring fat to induce gene mutations in bacteria was evaluated using the bacterial reverse mutation test. The bacterial reverse mutation assay, under the OECD guidelines, uses amino acid-requiring strains of *S. typhimurium* and *E. coli* to detect point mutations which involve substitution, addition or deletion of one or a few DNA base pairs, through the ability to functionally reverse mutations. The reverse mutations result in revertant colonies of bacteria with restored capability to synthesize the essential amino acid (*e.g.*, histidine and tryptophan). A mammalian microsome (S9 fraction) enzyme activation mixture was utilized in the bacterial culture system to facilitate the conversion of any potential promutagens into active DNA damaging metabolites.

The *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2 uvrA (Molecular Toxicology, Inc., Boone, NC) were used to determine the ability of the algal structuring fat to induce mutagenicity, either in the absence or presence of the S9 enzyme activation mixture (Molecular Toxicology, Inc., Boone, NC). The experiments utilized the standard plate incorporation method (Experiment I) and the pre-incubation method (Experiment II). The highest test substance concentration used in either study for all strains was 5000 μg/plate, based on preliminary experiments using strains TA98 and TA100 to determine test substance-induced cytotoxicity and, based on the OECD guideline-indicated limit concentration for this test. Six dose levels (1.58, 5.0, 15.8, 50, 158, 500, 1580 and 5000 μg/plate) were prepared by serial dilution in 0.1% (w/w) Tween-80 in 0.5% (w/v) aqueous methylcellulose (control vehicle) for Experiment I. Experiment II utilized eight dose levels (40, 80, 160, 320, 640, 1280, 2560 and 5000 μg/plate) in the same control vehicle. Tester strain concentrations of approximately $10^{\wedge}9$ cells/ml were used in the experiments (0.1 ml/plate). The positive control substances for these experiments in the absence of S9 were: sodium azide (NaN_3) for *S. typhimurium* strains TA100 and TA1535, ICR 191 acridine for *S. typhimurium* strain TA1537, daunomycin for *S. typhimurium* TA98, and MMS for *E. coli* strain WP2 uvrA. In the presence of S9, 2-aminoanthracene (2-AA) was utilized for all strains. All of the plates were prepared and evaluated in triplicate.

To be judged positive for mutagenicity, increases in the revertant colonies in the test plates would need to be biologically relevant (*i.e.*, greater than twice the negative control values for *S. typhimurium* TA98, TA100 and/or *E. coli* WP2 uvrA or greater than three times the control values for TA1535 and/or TA1537) and/or dose-dependent. Growth inhibition and the formation of precipitate were also reported, but not considered as mutagenic responses. No statistical analysis was conducted.

2.5.3. Chromosome aberration assay

The mammalian *in vivo* chromosome aberration assay was used to assess the ability of the algal structuring fat to promote clastogenic effects in the mouse model. Bone marrow is the target tissue because bone marrow is a highly vascularized tissue and contains rapidly cycling cells that are readily isolated and processed. The maximum tolerated dose (MTD) was determined to be 2000 mg/kg bw orally in a preliminary range-finding toxicity experiment. Based on the OECD guidelines, three oral dose

Table 5
Summary of mean terminal body and organ weights in female rats following dietary treatment with kokum fat or algal structuring fat.

Parameter	Group 1[a] 0 ppm (n = 19)	Group 2 25,000 ppm KF (n = 10)	Group 3 50,000 ppm KF (n = 10)	Group 4 100,000 ppm KF (n = 10)	Group 5 25,000 ppm AF (n = 10)	Group 6 50,000 ppm AF (n = 10)	Group 7 100,000 ppm AF (n = 10)
Mean terminal body and organ weights—day94/95							
Terminal body weight (g)	346.1 ± 43.4	344.3 ± 36.7	334.3 ± 43.9	349.7 ± 43.8	354.0 ± 38.6	338.5 ± 37.7	330.6 ± 28.3
Adrenals (g)	0.0729 ± 0.0120 I[2]	0.0770 ± 0.0087	0.0712 ± 0.0181	0.0583 ± 0.0147 D[1]	0.0711 ± 0.0085	0.0698 ± 0.0095	0.0623 ± 0.0142
Brain (g)	2.126 ± 0.084 I[2]	2.138 ± 0.073	2.128 ± 0.100	2.107 ± 0.063	2.137 ± 0.071	2.083 ± 0.100	2.082 ± 0.084
Heart (g)	1.117 ± 0.152 L[3]	1.128 ± 0.094	1.055 ± 0.122	1.090 ± 0.140	1.081 ± 0.112	1.057 ± 0.066	1.038 ± 0.088
Kidneys (g)	2.202 ± 0.240 R[4]	2.191 ± 0.205	2.213 ± 0.241	2.197 ± 0.499	2.179 ± 0.164	2.068 ± 0.133	2.150 ± 0.205
Liver (g)	9.025 ± 1.897 L[3]	8.745 ± 1.641	8.624 ± 1.504	9.191 ± 1.604	9.055 ± 0.907	8.388 ± 0.893	8.315 ± 0.857
Ovaries (g)	0.0772 ± 0.0187 I[2]	0.0897 ± 0.0159	0.0796 ± 0.0226	0.0814 ± 0.0253	0.0822 ± 0.0134	0.0804 ± 0.0215	0.0785 ± 0.0191
Spleen (g)	0.613 ± 0.096 I[2]	0.598 ± 0.054	0.595 ± 0.090	0.668 ± 0.100	0.652 ± 0.065	0.598 ± 0.086	0.604 ± 0.087
Thymus (g)	0.2750 ± 0.0549 L[3]	0.2872 ± 0.0629	0.2723 ± 0.1235	0.2997 ± 0.0781	0.2854 ± 0.0793	0.2929 ± 0.0928	0.2953 ± 0.0504
Uterus and Oviduct (g)	0.873 ± 0.355 L[3]	0.932 ± 0.302	0.822 ± 0.176	0.750 ± 0.180	0.760 ± 0.146	0.900 ± 0.351	0.742 ± 0.203
Organ-to-body weight ratios- Day94/95[b]							
Adrenals/TBW	0.2106 ± 0.0367	0.2245 ± 0.0230	0.2105 ± 0.0410	0.1683 ± 0.0413 D[1]	0.2032 ± 0.0340	0.2082 ± 0.0321	0.1889 ± 0.0404
Brain/TBW	6.221 ± 0.737 I[2]	6.266 ± 0.620	6.443 ± 0.702	6.117 ± 0.842	6.094 ± 0.612	6.199 ± 0.491	6.338 ± 0.587
Heart/TBW	3.208 ± 0.354 I[2]	3.292 ± 0.261	3.169 ± 0.229	3.134 ± 0.338	3.061 ± 0.205	3.140 ± 0.208	3.152 ± 0.280
Kidneys/TBW	6.381 ± 0.6789 R[4]	6.405 ± 0.702	6.646 ± 0.385	6.372 ± 1.717	6.203 ± 0.655	6.146 ± 0.440	6.519 ± 0.545
Liver/TBW	25.382 ± 2.918 L[3]	25.289 ± 2.742	25.724 ± 1.815	26.284 ± 3.357	25.677 ± 2.173	24.829 ± 1.470	25.174 ± 1.892
Ovaries/TBW	0.2248 ± 0.0618 I[2]	0.2647 ± 0.0575	0.2427 ± 0.0775	0.2356 ± 0.0745	0.2326 ± 0.0326	0.2354 ± 0.0449	0.2374 ± 0.0529
Spleen/TBW	1.794 ± 0.282 L[3]	1.747 ± 0.182	1.791 ± 0.248	1.925 ± 0.303	1.846 ± 0.115	1.767 ± 0.171	1.825 ± 0.193
Thymus/TBW	0.7822 ± 0.1486 R[4]	0.8331 ± 0.1619	0.8061 ± 0.3053	0.8541 ± 0.1759	0.8076 ± 0.2042	0.8652 ± 0.2611	0.8987 ± 0.1654
Uterus-Oviducts/TBW	2.591 ± 1.056 L[3]	2.744 ± 1.014	2.488 ± 0.574	2.167 ± 0.564	2.180 ± 0.528	2.675 ± 0.970	2.272 ± 0.675
Organ-to-brain weight ratios[b]							
Adrenals/BrW	0.0344 ± 0.0058 I[2]	0.0360 ± 0.0037	0.0334 ± 0.0082	0.0276 ± 0.0067	0.0333 ± 0.0043	0.0335 ± 0.0043	0.0300 ± 0.0073
Heart/BrW	0.525 ± 0.062 I[2]	0.528 ± 0.041	0.496 ± 0.053	0.517 ± 0.064	0.505 ± 0.042	0.508 ± 0.022	0.499 ± 0.038
Kidneys/BrW	1.036 ± 0.104 R[4]	1.024 ± 0.076	1.040 ± 0.107	1.040 ± 0.214	1.020 ± 0.068	0.993 ± 0.056	1.033 ± 0.097
Liver/BrW	4.242 ± 0.849 L[3]	4.086 ± 0.731	4.050 ± 0.658	4.365 ± 0.762	4.238 ± 0.410	4.025 ± 0.351	4.000 ± 0.455
Ovaries	0.0363 ± 0.0085 I[2]	0.0420 ± 0.0079	0.0374 ± 0.0106	0.0387 ± 0.0123	0.0384 ± 0.0057	0.0385 ± 0.0095	0.0376 ± 0.0085
Spleen/BrW	0.288 ± 0.041 I[2]	0.280 ± 0.022	0.279 ± 0.033	0.317 ± 0.044	0.305 ± 0.029	0.287 ± 0.036	0.290 ± 0.036
Thymus/BrW	0.1294 ± 0.0253 L[3]	0.1340 ± 0.0268	0.1283 ± 0.0584	0.1421 ± 0.0364	0.1336 ± 0.0365	0.1406 ± 0.0433	0.1423 ± 0.0268
Uterus-oviducts/BrW	0.412 ± 0.176 L[3]	0.438 ± 0.147	0.387 ± 0.083	0.355 ± 0.079	0.356 ± 0.068	0.431 ± 0.163	0.357 ± 0.101

AF = Algalfat; BrW = Brain weight; KF = Kokum fat; TBW = Terminal body weight.
1[D—Test Dunnett 2 sided $P < 0.05$]; 2[I—Automatic Transformation: Identity (No Transformation)]; 3[L—Automatic Transformation: Log]; 4[R—Automatic Transformation: Rank].
[a] Control group.
[b] Organ-to-body weight ratios and organ-to-brain weight ratio numbers are increased by a factor of 1000 for clarity.

levels (400, 1000 and 2000 mg/kg bw) of the algal structuring fat in corn oil were then used in the main study. The exposure times were 24 h and 48 h for the dose groups. Each test substance and control (corn oil) group included five male and five female mice, except for the 2000 mg/kg bw dose groups evaluated 48H after administration, which included seven males and seven females. Positive control mice (five mice/sex) received a single intraperitoneal dose of cyclophosphamide (CPA; 40 mg/kg bw). Four hours before euthanasia (*via* cervical dislocation), the metaphase-arresting agent Colcemid® (40 μg) was administered by intraperitoneal injection (i.p.) to all animals. Bone marrow was obtained from the femurs from treatment and negative control groups 24 and 48 h after test oil/negative control administration, and 24 h after treatment of the positive control group (n = five/sex). The bone marrow cells were fixed, stained and examined microscopically. Cytogenic damage was assessed by scoring 100 metaphases per animal for structural chromosome aberrations, such as breaks, fragments, deletions exchanges and chromosomal disintegrations. Gaps were recorded but not included in the aberration rate calculations. Cytotoxicity was evaluated by determining the mitotic index (% of cells in mitosis) for a minimum of 1000 cells per animal. If a definite and dose-related increase in aberration frequency occurred in the test substance-treated groups and the increase was also biologically relevant in at least one group (*i.e.*, greater than the laboratory negative control ranges of 0–5.0% aberrant cells in males and 0–3.0% aberrant cells in females), the assay would be judged positive for clastogenicity.

2.6. Statistical analyses

2.6.1. Thirteen-week dietary study

Mean and standard deviations were calculated for all quantitative data from the in-life, organ weights and clinical pathology parameters. All experimental groups were compared using a two-way analysis of variance (ANOVA). Data within groups were evaluated for homogeneity of variances and normality by Bartlett's test, when warranted by sufficient group sizes. Groups treated with the algal structuring fat or the kokum fat were compared with the control group by a variety of tests, including Dunn's test, Dunnett's test for multiple comparisons, Kruskal–Wallis nonparametric analysis of variance, when variances were considered significantly different by Bartlett's test. Where variances were considered significantly different by Bartlett's test, groups were compared using a nonparametric method (Kruskal–Wallis nonparametric analysis of variance). When non-parametric analysis of variance was significant, comparison of treated groups to control was performed using Dunn's test. Statistical analysis was performed using Provanits® version 9 (Instem LSS, Staffordshire, UK) and INSTAT Biostatistics, Graph Pad Software, San Diego, CA. Statistical significance was determined at the 5% level for all analyses.

2.6.2. Chromosome aberration assay

For statistical analysis, the Fisher's exact test was used with statistical significance determined at the 5% level. According to the OECD guideline, biological relevance was the primary consideration for the interpretation of the results. Statistical methods were used only as an aid in evaluating the test result.

3. Results

3.1. Test substance and diet preparation

The algal structuring fat was found to be stable under the suggested storage conditions, with a change of 4.6% compared to the neat substance (as determined by high performance liquid chromatography (HPLC)) for the overall test substance stability, which is within the range of analytical variance of the measured test substance. When mixed into the rat diet and sampled on Day -1 and after 4, 7 and 10 days of storage, the overall stability of the algal structuring fat was 98.4, 91.3 and 95.4% over a ten-day period for the nominal dietary concentrations of 25 000, 50 000 and 100 000 ppm algal structuring fat in the diet. In addition, a sampling from the top, middle and bottom of the dietary preparations found all dietary mixtures to be homogeneously distributed, with a relative standard deviation (RSD) of 2.1, 0.4 and 2.5% between the strata for the 25 000, 50 000 and 100 000 ppm algal structuring fat concentrations, respectively. Concentration verification studies showed that the algal structuring fat concentrations for Day -3 averaged 105.9, 102.7 and 96.8%; Day 39 resulted in 116.9, 105.5 and 99.8%; and Day 88 resulted in 120.6, 107.1 and 99.3% for 25,000, 50,000 and 100,000 ppm algal structured fat. The higher than expected recovery value in the low dose groups for concentration verification was not believed to have an impact on this study as the dose was well tolerated and there were no adverse observations for the toxicological endpoints evaluated.

Stability testing of the kokum fat test substance found a 7.4% difference between Day -3 (99.8%) and Day 84 (107.2%) values (as determined by HPLC analysis) under the conditions of the neat kokum storage, within the analytical variance range for the test substance (data not shown). Stability analysis of the kokum fat after 4, 7 and 10 days of storage when added to the diet found that the kokum in the diet was 101.4, 96.7 and 96.7% over the 10-day period for the nominal dietary concentrations of 25 000, 50 000 and 100 000 ppm kokum fat in the diet. The kokum fat was also found to be homogeneously distributed when sampled from the top, middle and bottom of the dietary preparations, with RSDs of 1.7, 2.0 and 1.3% for the concentrations of 25 000, 50 000 and 100 000 ppm kokum fat, respectively. Based on the stability, homogeneity and the concentration verification results, the rats were considered to have received the targeted concentrations of kokum fat.

3.2. Thirteen-week dietary study

No kokum fat- or algal structuring fat-related mortalities occurred during the study. One control male was found dead on Day 39 of the study, although no adverse clinical findings were noted prior to death. The cause of death could not be determined. Necropsy findings included a pale liver, multifocal and a mottled thymus due to hepatocellular vacuolization (lipid, presumptive) and thymic hemorrhage (agonal change). One control female was euthanized on Day 65 for humane reasons, as it was found to exhibit red bilateral ocular discharge, red nasal discharge, gasping, yellow ano-genital staining and a malocclusion of the upper incisors. There were no changes in clinical signs or detailed clinical observations associated with the treatment of kokum or algal structuring fat.

There were no changes in weekly body weight or body weight gain for the treatment groups in either male or female rats that were related to kokum fat or algal structuring fat administration (Figs. 1 and 2). Mean weekly and overall (Days 0–91) body weights and mean daily body weight gain for both male and female rats administered algal structuring fat were comparable to the control values for the same sex. The mean weekly body weights and the overall (Days 0–91) and mean daily body weight gain for the female rats administered kokum fat were comparable to values for female controls. The mean weekly body weights for the male rats administered kokum fat were comparable to values for control male rats. The overall (Days 0–91) and mean daily body weight gain of males provided kokum fat was also generally comparable with the control values, although a significant ($P < 0.05$) decrease in body weight gain was reported in the high dose kokum fat group on Days 70–77.

Overall (Days 0–91) and mean daily food consumption for both the male and female rats consuming the algal structuring fat were not statistically different from the control rats of the same sex. The overall (Days 0–91) and mean food efficiency for the male rats consuming the algal structuring fat were comparable with the control values. There were significant decreases in mean food efficiency in the 50 000 ppm male group on Days 28–35 and 70–77, and the male 100 000 ppm dose group on Days 49–56. For the females consuming the algal structuring fat, a significant ($P < 0.05$) decrease in mean food efficiency was reported in the 50 000 ppm dose group. Because decreased feed efficiency was transient, did not correlate with decreased body weight, and was not dose-dependent or observed in other male or female treatment groups, the decreases in food efficiency were considered incidental and not treatment-related. The overall (Days 0–91) mean daily intake of algal structuring fat in male rats fed dietary concentrations of 25 000, 50 000 and 100 000 ppm was 1285.6, 2594.3 and 5299.2 mg/kg bw/day, respectively. In female rats, the corresponding mean overall daily intake of algal structuring fat was 1606.0, 3069.7 and 6313.8 mg/kg bw/day, respectively. The overall (Day 0–91) mean daily intake of kokum fat in the male rats fed 25 000, 50 000 and 100 000 ppm was 1308.5, 2525.5, and 5247.3 mg/kg bw/day, respectively. For the female rats fed the same kokum concentrations, the overall mean daily intake was 1471.5, 3354.7 and 5943.7 mg/kg bw/day kokum fat, respectively.

Ophthalmoscopic examinations of both eyes of each rat were conducted prior to study initiation and on Day 88 of the study; one male in the 25 000 ppm algal structuring fat dose group had chorioretinal scarring in the left eye at Day 88. The appearance of the eye was comparable to a resolved vitreoretinal hemorrhage, a known sporadic occurrence in Sprague Dawley rats and was therefore considered incidental and not treatment-related. All other animals were normal on ophthalmic exam. There were no test substance-related changes in urinalysis parameters in male or female rats. No statistically significant differences between control and the exposed groups or between the different fats were reported for urinalysis parameters (data not shown).

Evaluation of hematology parameters from Day 86/88 (Tables 2 and 3) for the male and female rats fed algal structuring fat showed no test substance-related effects. There were no kokum fat-related changes in hematology parameters in male rats on Day 86. Hematology changes reported in female rats (Day 88) fed 100 000 ppm kokum fat included increases ($P < 0.05$) in platelet counts, absolute monocytes and absolute eosinophils in females only. Although the changes were statistically significant when compared to the control group, the changes were within the laboratory's historical control ranges for platelet counts ($502–1651 \times 103/\mu l$), absolute monocytes ($0.04–0.47 \times 10^3/\mu l$) and absolute eosinophils ($0.04–0.35 \times 10^3/\mu l$) and therefore within the expected biological variation for this strain and age of rat. There were no significant changes in overall white blood cell counts or any other hematological or histopathological correlates. The 50 000 ppm female kokum fat dose group exhibited a significant ($P < 0.05$) decrease in hemoglobin concentration compared to control females, although the value (14.6 ± 0.6 g/dl) was within the range of the laboratory's historical controls for this parameter (13.4–17.1 g/dl). Because this effect was not dose-dependent and was unaccompanied by any other corresponding clinical or histopathologic change, the result was considered not toxicologically relevant. There were no statistically significant differences

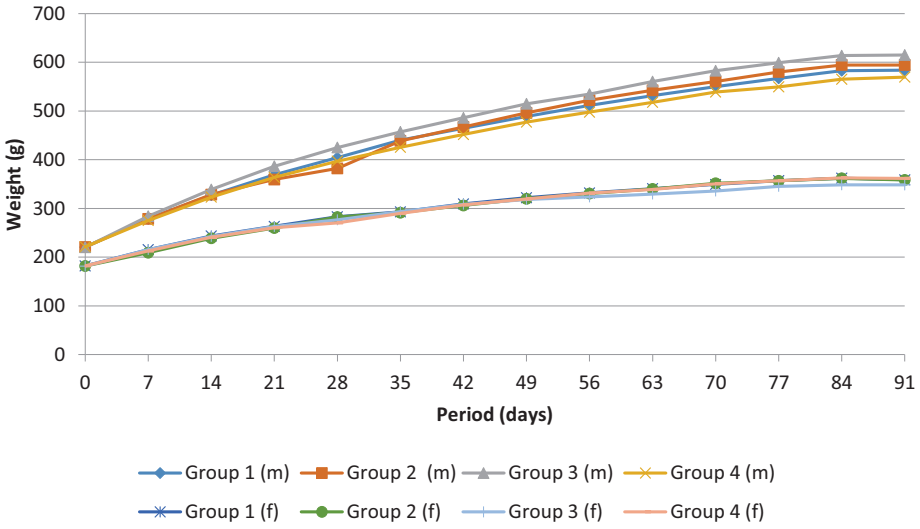

Fig. 1. Mean body weights of male (m) and female (f) rats consuming diets containing kokum fat.

in hematology parameters between the algal structuring fat and kokum fat test groups ($P > 0.05$).

Clinical chemistry values were not different between male (Table 2) or female (Table 3) rats treated with kokum fat, when compared to the corresponding control group. A decrease in blood urea nitrogen (BUN) was observed in the 100 000 ppm algal structuring fat male dose group; this slight but significant ($P < 0.05$) decrease was within the contract laboratory's historical control range (7–24 mg/dL) and was not toxicologically relevant. When comparing results from the algal structuring fat dose groups to the kokum fat dose groups, BUN values in males at the 100 000 ppm algal structuring fat dose group were lower than values for corresponding dose of kokum fat in the same sex ($P < 0.05$). However, all values were within the laboratory historical control range (7–24 mg/dl) and were considered as being within biological variation for this strain and age of rats.

There were no macroscopic findings that were considered related to exposure to either the algal structuring fat or to the kokum fat, in either the male or female rats. Fluid-filled uteri at necropsy were found the females of the control group (7/19), the 25 000 ppm kokum group (6/10), and the 100 000 ppm algal structuring fat group (3/10). The fluid filled uteri usually corresponded microscopically to luminal dilation of the uterus, attributable to variations in the estrous cycle in individual animals. As such, this finding was not associated with test substance administration. The remaining macroscopic observations at study termination were of sporadic incidence and were not related to any trends/patterns that suggested a relationship to administration of either the algal structuring fat or kokum fat. The findings included mild dermal edema in the ear of one 25 000 ppm kokum fat group male; a 25 000 ppm kokum fat group female with adipose tissue focus, retroperitoneum, tan, increased firmness, irregular shape; and a 50 000 ppm kokum fat group male with adipose tissue focus, mesentery, round, yellow, firm, increased firmness, 2×10 mm, both due to focal steatitis; a 100 000 ppm algal structuring fat group male with epididymides nodule, right of the tail, yellow, 3×3 mm due to focal sperm granuloma; a 25 000 ppm algal structuring fat group male with testes soft, left, and epididymides small, left; a 50 000 ppm algal structuring fat group male with testes soft, left, and epidiymides small, left; a 100 000 ppm algal structuring fat group male with testes small, bilateral and epididymides small, bilateral, and another male in the same group with an enlarged

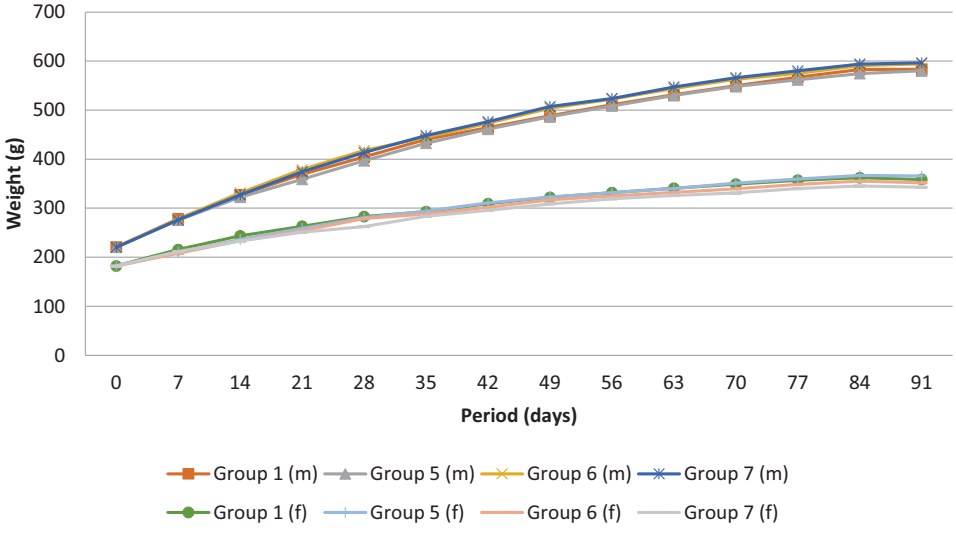

Fig. 2. Mean body weights of male (m) and female (f) rats consuming diets containing algal structuring fat.

right testes, 18 × 9 mm, a small left testes (25 × 12 mm) and epididymides small, right. All small/soft testes were due to germ cell degeneration and small epididymides were due to aspermia or oligospermia and the enlarged testis did not correlate to any histologic finding.

A 100 000 ppm kokum fat group female presented with a pale, diffuse liver with an irregular surface, which correlated to marked bile duct ectasia, and kidney focus, bilateral, tan, multifocal, pinpoint with an irregular bilateral, multifocal, diffuse surface due to cysts in the kidneys. A 100 000 ppm algal structuring fat group male had thymus discoloration, multifocal with redness due to agonal hemorrhage. In addition, macroscopic findings occurred which did not have histologic correlates, including a 100 000 ppm algal structuring fat group male with a small left adrenal gland lobe, a 25 000 ppm kokum fat group female with a liver focus on the right lateral lobe, 3 × 6 mm; a 50 000 ppm kokum fat group male with a liver focal area indentation on the left lateral lobe (7 mm), and a 50 000 ppm algal structuring fat group female with lung discoloration (red) on the left lobe at the cranial aspect (1.5 × 2 cm). There were no microscopic findings that were considered related to exposure to either test substance.

The absolute mean organ weights and mean organ-to-body and organ-to-brain weight ratios for the male rats that consumed either the algal structuring fat or kokum fat were not statistically different when compared with the control animals (Table 4). There were no algal structuring fat-related changes in absolute or relative organ weight changes in either the male or female rats that were considered to be adverse (Table 4, Table 5). Significant decreases in the liver-to-body weight ratios for the 50 000 and 100 000 ppm algal structuring fat male dose groups occurred compared to control males, but these changes were of small magnitude, were not reflected in corresponding decreases in absolute or liver-to-brain weight, and lacked histopathological or serum chemistry correlates and were therefore of no toxicological relevance. There were potentially test substance-related decreases ($P < 0.05$) in the absolute and relative adrenal gland -to-body weight parameters in the 100 000 ppm kokum fat group females (Table 5). However, these changes were not associated with histomorphological adrenal findings and were therefore interpreted to be non-adverse in nature.

3.3. Bacterial reverse mutation assay

No toxic effects of the algal structuring fat were observed in any strain at any dose level. Adequate background lawn growth was noted on both the control and algal structuring fat-treated plates. Microbial contamination occurred in one of the three plates at the 50 μg/plate concentration for strain TA1535 in the main test; this contamination did not affect the validity of the study. Each strain treated with the vehicle control provided mean revertant colony counts that were within the laboratory historical control range and/or published values [14,8]. The positive control substances caused the expected substantial increases in revertant colony counts in both the absence and present of the S9 mix in the main test. In the confirmatory test, the values for the positive control for the *E. coli* strain were adequate when evaluated without S9 activation, but were slightly lower than the expected two-fold increase when evaluated with S9 activation. The same positive control showed an adequate response in both the presence and absence of S9 in the main test (data not shown) and therefore, each phase of the test was considered valid.

The algal structuring fat was solid at room temperature, although maintaining the test substance temperature at 45 °C during preparation allowed the test substance to be miscible with the vehicle. Even so, a minimal to heavy precipitate was observed at dose levels ≥40 μg/plate. The precipitate was present in a dose-dependent manner and was due to the solid nature of the test substance at assay temperature (*i.e.*, a solid fat at room temperature). However, as the average numbers of revertant colonies for all strains that were incubated with the algal structuring fat (with or without S9 activation) in both the main and confirmatory tests were similar to the revertant colony averages for the vehicle controls, it was determined that the precipitate did not alter the system such that it impacted the determination of mutagenicity. Concentrations of 1.58, 5 and 15.8 μg/plate did not result in precipitate formation, providing an appropriate concentration range to evaluate mutagenicity. There were no concentration-related or substantial, biologically relevant test substance-related increases in the number of revertant colonies (*i.e.*, greater than twice the negative control for TA98, TA100 or WP2 uvrA or greater than three times the control values for TA1535 and/or TA1537) observed by the algal structuring fat in any of the bacterial strains tested either in the absence or presence of the S9 metabolic activation mix (data not shown), when compared to the historical vehicle control data for these test strains. The control responses in these strains (without S9 mix) were: TA1535 (11–16), TA1537 (7–15), TA98 (26–35), TA100 (104–117) and WP2 uvrA (24–43) and, with S9 mix the control ranges were: TA1535 (9–15), TA1537 (9–19), TA98 (38–50), TA100 (87–123) and WP2 uvrA (38–55). Under the conditions of the study, the algal structuring fat did not cause gene mutations by base pair changes or frame shifts in the susceptible genes of the bacterial strains in this study.

3.4. Chromosomal aberration assay

Exposure to a single oral dose of algal structuring fat for 24 or 48 h did not affect the mean number of chromosomal aberrations in the bone marrow of the mice of either sex (Table 6). The mean values noted for the algal structuring fat dose levels of 400, 1000 and 2000 mg/kg bw in the mice (exposed for 24 and 48 h) were within the historical control data range (0.0–5.0% for male mice and 0.0–3.0% for female mice). The mitotic index was determined for each animal and the mean mitotic index was determined for each test group, which is a supportive endpoint to assess cytotoxicity. Male mice exposed to 2000 mg/kg bw for 24 h showed mean aberrant cell values of 0.2% and female mice exposed for either 24 or 48 h exhibited no aberrant cells (0.0%). There was no dose-dependent, biologically relevant increase in chromosomal aberrations following treatment with the algal structuring fat (Table 6). For verification, the Fisher's exact statistical test was performed, which showed that no statistically significant ($P > 0.05$) change was found when comparing the number of chromosomal aberrations from the vehicle-dosed animals to those from the algal structuring fat-dosed animals.

4. Discussion

Consumption of either the algal structuring fat or kokum fat mixed into the diet for 13 weeks was well-tolerated by the Sprague-Dawley (SD) CD® IGS rats at all dietary concentrations. There were no reported treatment-related adverse events in any treatment group. One control group male rat was found dead on Day 39 with no adverse clinical findings noted prior to death, and one control female was euthanized on Day 65, which exhibited red bilateral ocular discharge, red nasal discharge, gasping, yellow ano-genital staining and a had a malocclusion of the upper incisors. No deaths occurred that were attributable to administration of either test substance. Daily ingestion of either test fat did not affect any monitored health or growth parameter, including behavior, appearance, body weight or weight gain, food consumption or food efficiency. Standard preclinical toxicity endpoints evaluated in this dietary study included ophthalmology, urinalysis, hematology, clinical chem-

Table 6
Summary of chromosome aberration assay results for algal structuring fat.

Study groups ($n = 5$)	Metaphases	Aberrant cells[a] (total/% ± SD)	Mean mitotic index	Mean body weight (g ± SD)
Negative control, 24 h				
Male	500	0/0.0 ± 0.0	7.66	33.2 ± 1.6
Female	500	0/0.0 ± 0.0	8.04	28.3 ± 0.8
Positive control, 24 h				
Male	250[b]	185/74.0 ± 12.3*	1.28	34.3 ± 2.0
Female	300[c]	107/35.7± 4.9*	1.52**	28.6 ± 1.5
Treatment group (0.2 MTD), 24 h				
Male	500	1/0.2 ± 0.4	6.32	35.4 ± 2.4
Female	500	1/0.2 ± 0.4	7.36	27.5 ± 0.9
Treatment Group (0.5 MTD), 24 h				
Male	500	1/0.2 ± 0.4	6.66	33.9 ± 1.0
Female	500	0/0.0 ± 0.0	7.78	28.2 ± 0.9
Treatment group (1.0 MTD), 24 h				
Male	500	1/0.2 ± 0.4	8.44	34.3 ± 2.1
Female	500	0/0.0 ± 0.0	9.50	29.1 ± 1.2
Negative control, 48 h				
Male	500	1/0.2 ± 1.6	6.26	34.0 ± 1.1
Female	500	1/0.2 ± 0.4	9.02	28.6 ± 1.9
Treatment group, 48 h				
Male	500	1/0.2 ± 0.4	8.16	35.4 ± 1.5
Female	500	0/0.0 ± 0.0	9.36	27.1 ± 0.7

MTD = Maximum tolerated dose; SD = Standard deviation.
* $P < 0.01$, *vs.* corresponding 24-h negative control group.
** $P < 0.05$, *vs.* corresponding 24-h negative control group.
[a] Aberrant cells, excluding chromosomal gaps.
[b] Five mice (50 metaphases).
[c] Four mice (50 metaphases), one mouse (100 metaphases).

istry, gross pathology, organ weights or histopathology. There was a slight but statistically significant decrease in the liver:total body weight ratios for the male rats that consumed the algal structuring fat; however, there was no significant changes in liver:brain weight ratios for these groups. The body weights for the 50,000 ppm and 100,000 ppm algal structuring fat dose groups were slightly increased, resulting in the decreased liver:body weight ratios. Macroscopic observations at study termination were of sporadic incidence and were not related to any trends/patterns or of a statistically significant or dose-dependent nature that suggested a relationship to administration of either the algal structuring fat or kokum fat. Any changes to the testes of the male rats consuming the test articles did not correlate with any microscopic or histological findings and was not dose-dependent or statistically significant; therefore, was not considered a causal effect of test article consumption. The NOAEL for both the algal structuring fat and the kokum fat was 100 000 ppm, as determined through evaluation of the toxicological endpoints of this 13-week dietary study. The 100 000 ppm level was the highest dose tested, and was equivalent (on a mg/kg bw/day basis) to a dietary NOAEL for the algal structuring fat of 5299.2 mg/kg bw/day and 6313.8 mg/kg bw/day in the male and female rats, respectively, and a dietary NOAEL for the kokum fat of 5247.3 mg/kg bw/day and 5943.7 mg/kg bw/day in the male and female rats, respectively.

The absence of toxicity seen when this novel structuring fat produced by a genetically engineered microalgae was used in this study, is consistent with the evaluation of other microalgal-derived products in subchronic dietary studies, including the 13-week subchronic study of a DHA-rich oil derived from *Schizochytrium* sp. at up to 50 000 ppm in the diet (equivalent to 3305 and 3679 mg/kg bw/day in male and female rats, respectively) [17], a 13-week dietary study of a high lipid-containing whole algal flour product from the biomass of *C. protothecoides* (which resulted in the determination of a NOAEL of 4807 mg/kg bw/day in male rats and 5366 mg/kg bw/day in female rats) [21], a 13-week dietary study of a whole algal protein product from the high-protein biomass of *C. protothecoides* (which resulted in the determination of a NOAEL of 4805 mg/kg bw/day in male rats and 5518 mg/kg bw/day in female rats) [21], a 13-week dietary study of a high oleic acid-containing oil produced from a genetically engineered strain of *P. moriformis* (NOAEL of 5200 and 6419 mg/kg bw/day in male and female rats, respectively) [22], and a 13-week oral gavage study of a high eicosapentaenoic acid (EPA)-containing ingredient from *Nannochloropsis oculata* at up to 2000 mg/kg bw/day [11]. In all of the studies referenced, the microalgal test substance was well tolerated at the highest dose tested; the NOAEL determined from each study was based on the highest dose provided to the animals. These studies demonstrate the safety of these food ingredients derived from microalgae. Similar to the different microalgae-based substances discussed above, the series of toxicity studies described here confirms that the structuring fat produced from a uniquely engineered strain of *P. moriformis* is not toxic when administered in the diet of rats fed for 13 weeks.

The algal structuring fat examined in the *in vitro* bacterial reverse mutation assay did not induce mutations in the *S. typhimurium* and *E. coli* strains utilized in the study, when evaluated at concentrations up to 5000 μg/plate, even when precipitation was formed at ≥40 μg/plate. Although high fat concentrations have been shown to alter the total fatty acid composition of *S. typhimurium* cells [3], no alterations from historical control responses were seen in the current study, indicating that the addition of the algal structuring fat did not adversely impact the validity of the study. In addition, oral administration of the algal structuring fat to mice did not induce clastogenic effects in the bone marrow, a highly vascular tissue with a high production of new cells that increases the probability of finding genotoxic responses. This is consistent with the lack of mutagenic or clastogenic effects by a high-oleic acid oil produced by *P. moriformis* [22], a high-lipid

whole algal flour from *C. protothecoides* [20], a whole algal protein from *C. protothecoides* [21], or oils produced from *Schizochytrium* sp., *Ulkenia* sp. or *N. oculata* [2,17].

In summary, the evaluation of an algal structuring fat produced by a unique *P. moriformis* microalgae in a 13-week dietary toxicity study, as well as studies on the potential of the test substance to induce mutagenic or clastogenic effects, support the safety for this algal structuring fat produced using an algal heterotrophic fermentation process for use in food. The 13-week toxicity study also found that the consumption of kokum fat, a substance with a fatty acid profile and TAG structure similar to the algal structuring fat, but which is already consumed in the EU and India, showed no toxicity in SD rats when fed at up to 100 000 ppm in the diet. Based on the 13-week study, the NOAEL for the algal structuring fat was 5299.2 mg/kg bw/day in male and 6313.8 mg/kg bw/day in female rats, and for the kokum fat the NOAEL was 5247.3 mg/kg bw/day in male and 5943.7 mg/kg bw/day in female rats, the highest doses evaluated.

This is the second study demonstrating the safety of substances derived from genetically engineered strains of *P. moriformis*. In the first study, a 13-week dietary study of high oleic oil from a different genetically engineered *P. moriformis,* the NOAEL was also 100 000 ppm, which was the highest concentration tested and there were no mutagenic or clastogenic effects produced by the oil [22]. In the current study, the algal structuring fat produced by an engineered strain of *P. moriformis* had similar safety results. Collectively, these studies on the oils produced from different, genetically engineered strains of *P. moriformis* show that these oils, despite significant differences in their fatty acid composition, TAG compositions and physical properties, support the safety of microalgae oils for replacement or complementation of vegetable and animal oils and fats in the human diet.

Conflict of interest

All authors have a financial relationship with the sponsor of the studies and manuscript, Solazyme, Inc., South San Francisco, CA.

Transparency document

The http://dx.doi.org/10.1016/j.toxrep.2015.12.006 associated with this article can be found in the online version.

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