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Safety assessment of a lyophilized biomass of *Tetraselmis chuii* (TetraSOD®) in a 90 day feeding study

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ABSTRACT

TetraSOD® is a powder of the lyophilized biomass of *Tetraselmis chuii strain CCFM03,* a marine microalga with a history of use as feed in the aquaculture industry. Recently, algae including *T. chuii* have been investigated for their potential use in human food. However, published toxicology studies addressing the safety of *T. chuii* as a food ingredient are not available. To address this issue, the toxicity of TetraSOD® was evaluated using a 90-day oral toxicology study in rats following the Organisation for Economic Co-operation and Development (OECD) test guideline 408. No treatment-related mortality or clinical signs were noted with TetraSOD® at doses of 625, 1667, or 2500 mg/kg/day. Additionally, no adverse effects on haematology, blood biochemistry, organ weights, gross or histopathology were observed. The Non Observed Adverse Effect Level (NOAEL) for TetraSOD® is greater than the highest tested dose of 2500 mg/kg/day.

1. Introduction

Tetraselmis chui Butcher (1959), more commonly spelled *Tetraselmis chuii*, is a unicellular, marine microalga belonging to the class Prasinophyceae. It was first isolated off the coast of Great Britain in the 1950s (Butcher, 1959) but has since been found around the world. Like other species of microalgae, *T. chuii* has a long history of use as feed for fish, crustacean, and mollusk larvae in the aquaculture industry (Lavens and Sorgeloos, 1996; Muller-Feuga, 2000) and food industry (Bonilla-Ahumada et al., 2018). *T. chuii* does not produce toxins and is innocuous toward marine species (Spolaore et al., 2006; Hallmann, 2007). It is also easy to culture and has a high nutritional value (Brown et al., 1999; Mourente et al., 1990).

Microalgae have gained increased interest in recent years for their potential as a sustainable and nutrient-rich food source (Enzing et al., 2014). Spirulina, a cyanobacteria, has been designated a "super food" by the World Health Organisation (WHO) and is being used to fight severe malnutrition worldwide (IIMSAM, 2019). *Chlorella* is another microalga used in human food due to its high content of protein, carotenoids, vitamins, and minerals (Liu and Chen, 2014). Finally, *Euglena* and its characteristic beta-glucan, paramylon, have recently undergone safety testing for their use in human food (Simon et al., 2016; Symonds et al., 2018). Spirulina dried biomass and *Chlorella*-derived macronutrients have been determined to be Generally Recognized as Safe (GRAS) (spirulina: GRAS notifications (GRNs) 127, 394, and 417; *Chlorella*-derived products: GRNs 384, 469, and 519) (FDA, 2003; FDA, 2012a; FDA, 2012b; FDA, 2012c; FDA, 2013; FDA, 2014). TetraSOD® is a unique commercial product comprised of 100% lyophilized *T. chuii strain CCFM03* biomass, that is currently marketed for food and nutraceutical applications around the world. Fitoplancton Marino, SL (El Puerto de Santa Maria, Spain) has developed a process to produce this microalgae strain with a high content of the enzyme superoxide dismutase (SOD) (patent pending).

T. chuii has a nutrient profile similar to that of other microalgae and thus offers many of the same potential benefits for use in food. It is composed mainly of proteins, carbohydrates, and fats (Becker, 2007). All essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) are present, making *T. chuii* a source of complete protein (Barka and Blecker, 2016). This species is also rich in long-chain polyunsaturated fatty acids (PUFA), especially omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Dunstan et al., 1992), as well as fat-soluble carotenoids (Brown and Jeffrey, 1992; Safafar et al., 2015; Takaichi, 2011). *T. chuii* contains 35–40% protein (including all the essential amino acids), 5–10% fat, 30–35% carbohydrate, and 15% ash (AESAN, 2013).

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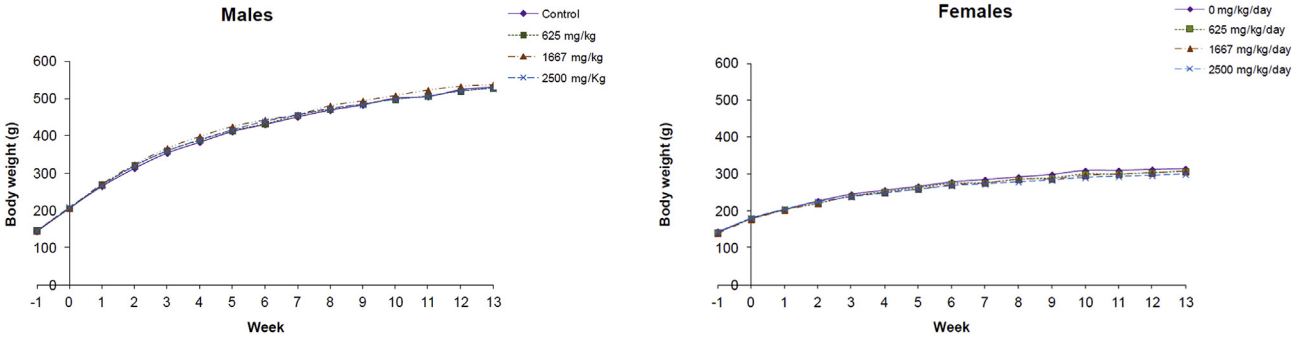

Fig. 1. Body weights of male and female rats administered vehicle control or TetraSOD® (625, 1667 or 2500 mg/kg/day) by oral route for 90 days. Data are presented as mean ± standard deviation (n = 10/sex/group).

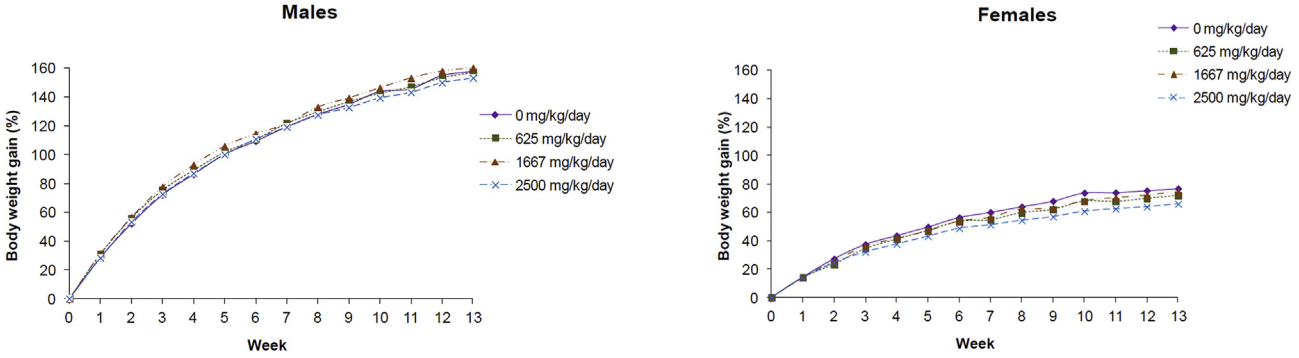

Fig. 2. Mean body weight gain (%) of male and female rats exposed to TetraSOD® (625, 1667 or 2500 mg/kg/day) by oral route and control rats for 90 days.

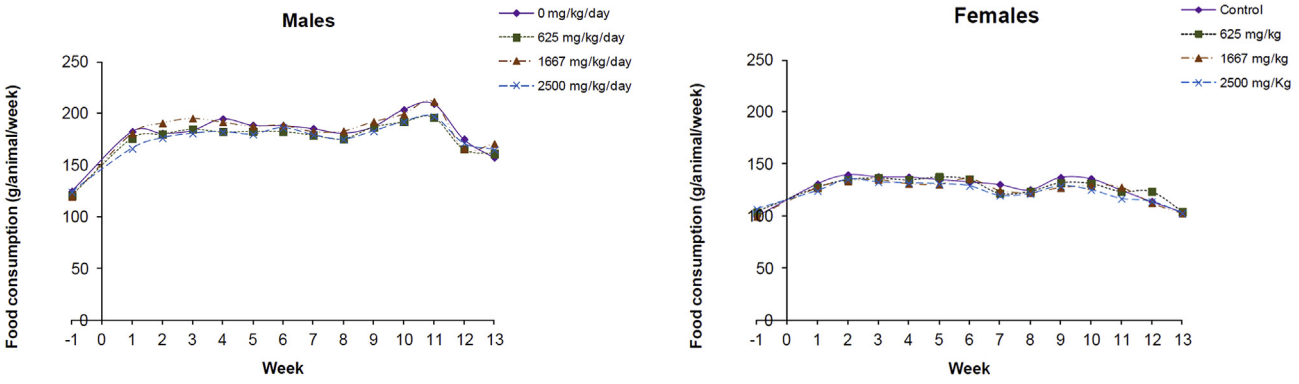

Fig. 3. Food consumption pattern (g) of male and female rats administered to TetraSOD® (625, 1667 or 2500 mg/kg/day) by oral route and control rats for 90 days.

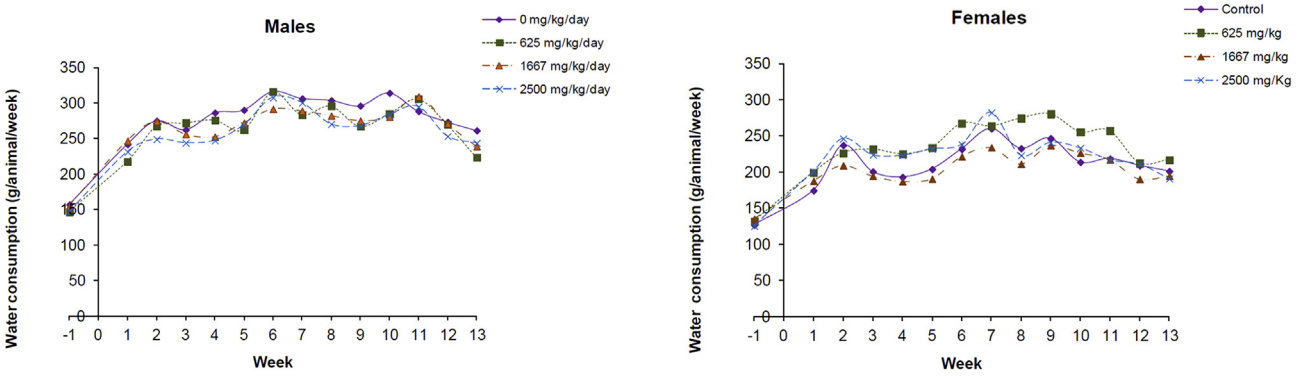

Fig. 4. Water consumption pattern (g) of male and female rats administered to TetraSOD® (625, 1667 or 2500 mg/kg/day) by oral route and control rats for 90 days.

Table 1
Haematology parameters of male and female rats fed with different doses of TetraSOD® in the diet for 90 d. Values are mean ± SD for 10 rats/sex/group. The differences between control and male and female exposed rats were evaluated by K.W:Kruskal-Wallis test (K.W.) or by ANOVA test (F values).

HAEMATOLOGY DATA SUMMARY									
		MALE				FEMALE			
		Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
		(0 mg/kg/day)	**(625 mg/kg/day)**	**(1667 mg/kg/day)**	**(2500 mg/kg/day)**	**(0 mg/kg/day)**	**(625 mg/kg/day)**	**(1667 mg/kg/day)**	**(2500 mg/kg/day)**
		N = 10	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**
RBC	**MEAN**	8.64	8.50	8.27	8.41	8.18	8.07	8.21	8.09
10^6/µl	**ST. DEV.**	0.31	0.34	0.31	0.59	0.42	0.71	0.35	0.35
		F(36.3) = 1.53 p = 0.22; N.S.				**F(36.3) = 0.16 p = 0.92; N.S.**			
HGB	**MEAN**	14.4	14.2	13.9	14.2	13.8	13.6	13.9	13.7
g/dL	**ST. DEV.**	0.6	0.6	0.6	0.8	0.6	1.1	0.5	0.2
		KW = 1.55 p = 0.67; N.S.				**F(36.3) = 0.36 p = 0.78; N.S.**			
HCT	**MEAN**	70.0	70.0	69.0	68.0	70.0	69.0	70.0	68.0
%	**ST. DEV.**	3.0	4.0	3.0	4.0	4.0	6.0	2.0	2.0
		F(36.3) = 0.54 p = 0.66; N.S.				**KW = 2.74 p = 0.43; N.S.**			
MCV	**MEAN**	80.8	81.0	82.4	81.9	86.3	85.2	85.2	85.3
fL	**ST. DEV.**	2.2	2.3	2.4	5.2	2.4	2.6	2.6	2.9
		F(36.3) = 0.51 p = 0.67; N.S.				**F(36.3) = 0.39 p = 0.76; N.S.**			
MCH	**MEAN**	16.7	16.7	16.9	16.9	17.1	17.3	17.2	17.2
pg	**ST. DEV.**	0.7	0.5	0.5	0.8	0.4	0.7	0.6	0.4
		F(36.3) = 0.41 p = 0.74; N.S.				**F(36.3) = 2.50 p = 0.08; N.S.**			
MCHC	**MEAN**	20.5	20.6	20.7	20.7	19.8	20.1	20.0	20.1
g/dL	**ST. DEV.**	0.5	0.3	0.8	0.5	0.3	0.5	0.3	0.3
		F(36.3) = 0.24 p = 0.87; N.S.				**F(36.3) = 1.46 p = 0.24; N.S.**			
PLT	**MEAN**	945.0	1067.0	797.0	988.0	1167.0	811.0	888.0	865.0
10^3/µl	**ST. DEV.**	208.0	239.0	263.0	204.0	640.0	203.0	88.0	204.0
		F(36.3) = 2.42 p = 0.08; N.S.				**KW = 7.70 p = 0.05; N.S.**			
APTT	**MEAN**	44.0	44.2	44.6	45.3	42.0	43.4	42.9	41.1
Sec.	**ST. DEV**	4.3	7.1	3.8	3.7	1.4	3.2	2.1	1.4
		KW = 1.69 p = 0.64; N.S.				**KW = 7.24 p = 0.06; N.S.**			
PT	**MEAN**	19.5	19.7	19.6	19.3	18.3	18.4	18.7	18.7
Sec.	**ST. DEV**	0.5	0.3	0.6	0.7	0.6	0.7	0.6	0.9
		F(36.3) = 1.10 = 0.36; N.S.				**KW = 5.35 p = 0.14; N.S.**			
RETIC.	**MEAN**	1.5	1.6	1.5	1.6	1.4	1.4	1.6	1.5
%	**ST. DEV.**	0.7	1.1	0.7	1.0	0.7	0.7	0.7	0.7
		KW = 0.06 p = 0.99; N.S.				**KW = 0.88 p = 0.82; N.S.**			

RBC: Erythrocyte count; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet (thrombocyte) count; APTT: activated partial thromboplastin time; PT: prothrombin time; RETIC: reticulocytes; K.W:Kruskal-WallisStatistic; N.S.: Not Significant; *Significantly different from control. *when $p < 0.05$; F: Statistics ANOVA test.

Not surprisingly, there is increasing interest in the use of unicellular microalgae as a source of antioxidants (Borowitzka, 2013; Banskota et al., 2019; Goiris et al., 2012; Natrah et al., 2007; Rodriguez-Garcia and Guil-Guerrero, 2008; Chacon-Lee and Gonzalez-Guerrero, 2010; Li et al., 2008). Carotenoids and phenolic compounds have been shown to be important contributors to antioxidant activity in microalgal biomass (Goiris et al., 2012). *T. chuii* was shown to contain total phenolics of 3.74 ± 0.10 mg GAE/g dry weight, including 2.88 ± 0.29 mg carotenoids (Goiris et al., 2012). Moreover, among 9 microalgae tested (*Botryococcus braunii*, *Chlorella sorokiniana*, *Nannochloropsis granulate*, *Neochloris oleoabundans*, *Phaeodactylum tricornutum*, *Porphyridium aerugineum*, *Scenedesmus obliquus*, *Scenedesmus sp.*, and *T. chuii*), an extract of *T. chuii* had the highest total phenolic content. The *T. chuii* extract was tested for various carotenoids and was shown to contain astaxanthin, lutein, zeaxanthin, cantaxanthin, á-carotene, â-carotene, lycopene, and others; together carotenoids comprised 8.5 mg/g dry weight (Banskota et al., 2019). Among tested microalgae, *T. chuii* extract also showed the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging potency of 45.0% DPPH of radicals at 200 ìg/mL. The oxygen radical absorbance capacity (ORAC) value of 2121 ìmol trolox equivalents (TE)/100 g was also notable (Banskota et al., 2019). In another study, ethanol/water extracts of *Tetraselmis* sp. were shown to have high antioxidant activity as measured by ferric reducing antioxidant potential (FRAP), trolox equivalent antioxidant capacity (TEAC), and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH-induced) oxidation of linoleic acid (AIOLA) assays (Goiris et al., 2012). Clearly, *T. chuii* is a promising potential source of antioxidants.

The European Union (EU) approved the company Fitoplancton Marino to commercialize its lyophilized *T. chuii* biomass as a novel food in 2014, in accordance with Article 3(1) of Regulation (EC) No 258/97 (AESAN, 2013). In 2017, the EU also approved the company to commercialize its lyophilized *T. chuii* biomass for use in food supplements as TetraSOD®, at levels up to 250 mg/day (AECOSAN, 2017). Other food categories lyophilized *T. chuii* that have authorization are sauces,

Table 2
Total and differential leukocyte count results after 90 days of TetraSOD® administration. Data are presented as mean ± SD (n = 10/sex/group). The differences between control and male and female exposed rats were evaluated by K.W:Kruskal-Wallis test (K.W.) or by ANOVA test (F values).

DIFFERENTIAL WHITE BLOOD CELLS COUNT DATA SUMMARY									
		MALE				FEMALE			
		Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
		(0 mg/kg/day)	**(625 mg/kg/day)**	**(1667 mg/kg/day)**	**(2500 mg/kg/day)**	**(0 mg/kg/day)**	**(625 mg/kg/day)**	**(1667 mg/kg/day)**	**(2500 mg/kg/day)**
		N = 10	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**
WBC	**MEAN**	7.52	7.47	7.57	7.58	5.65	5.93	5.24	4.05
10^3/μL	**ST. DEV.**	1.00	1.30	1.99	2.46	2.52	1.53	1.64	1.46
		F(36.3) = 0.01 p = 0.99; N.S.				**F(36.3) = 2.02 p = 0.12; N.S.**			
NE	**MEAN**	13.67	17.00	15.52	15.24	13.35	11.27	13.97	12.42
%	**ST. DEV.**	4.31	9.22	6.2	5.08	7.03	2.37	6.51	2.35
		KW = 0.45 p = 0.92; N.S.				**KW = 1.26 p = 0.73; N.S.**			
LY	**MEAN**	77.41	74.10	75.08	75.60	78.46	82.84	78.38	80.32
%	**ST. DEV.**	7.61	11.36	7.07	6.94	9.47	3.39	8.12	3.72
		KW = 1.07 p = 0.78; N.S.				**KW = 2.18 p = 0.53; N.S.**			
MO	**MEAN**	5.99	4.61	6.46	6.16	4.43	3.29	4.11	4.01
%	**ST. DEV.**	3.42	2.71	2.31	1.97	1.52	2.10	1.78	1.45
		KW = 2.66 p = 0.44; N.S.				**F(36.3) = 0.78 p = 0.51; N.S.**			
EO	**MEAN**	2.28	3.28	2.29	2.24	2.99	1.94	2.75	2.25
%	**ST. DEV.**	0.98	1.46	0.82	1.06	1.37	0.61	1.26	0.61
		F(36.3) = 2.09 p = 0.11; N.S.				**F(36.3) = 2.16 p = 0.10; N.S.**			
BA	**MEAN**	0.65	0.47	0.66	0.74	0.76	0.68	0.78	1.00
%	**ST. DEV.**	0.48	0.54	0.38	0.59	0.52	0.44	0.49	0.61
		KW = 3.69 p = 0.29; N.S.				**F(36.3) = 0.72 p = 0.54; N.S.**			

WBC: total leukocyte count; NE: neuthrophil; LY: lymphocyte; MO:monocyte; EO:eosinophil; BA:basophil.
N.S.: Not significant.

special salts and condiment (Reg. EU, 2017/2470). Fitoplancton Marino's lyophilized *T. chuii* was also recently approved for novel food use in Canada (Health Canada, 2018).

At European level, the number of microalgae approved as novel food is still scarce, although algae-derived products are more common. Thus, currently in the Union List (Reg. EU, 2017/2470), apart from freeze-dried *T. chuii,* only the microalga *Odontella aurita* has been also included. This one can be used in different food categories such as flavoured pasta (1.5%) or fish soup (1%).

Moreover, recent studies have focused on the use of *Tetraselmis sp.* as well as other microalgae like *Spirulina sp.*, and *Chlorella sp.*, to enrich innovative food commodities (broccoli soup) (Lafarga et al., 2019), suggesting the extensive potential use of this microalga.

One of the main toxicological requirements for the approval of a novel food is to perform a subchronic toxicity study in order to provide information on health hazard likely to arise from exposure to a test substance via oral administration (Regulation EC 258/97; Regulation EU, 2015/2283; Canada Food and Drug Regulation Section B28.002). Thus, the purpose of the current study was to assess the safety of TetraSOD® via a 90-day repeated dose toxicology study in rats.

2. Materials and methods

2.1. Test substance

TetraSOD® is a green lyophilized powder comprised 100% of the marine microalga *Tetraselmis chuii (T. chuii) strain CCFM 03*. TetraSOD® was provided by Fitoplancton Marino S.L. (El Puerto de Santa María, Cádiz, Spain). This product is also marketed under the brand name Oceanix™ in the United States and Canada (Lonza, 2019). This microalga is grown in controlled outdoor close photobioreactors (proprietary process) under photoautotrophic conditions which yields a product with high superoxide dismutase (SOD) content (patent pending), showing a SOD activity higher than 180 IU/mg soluble protein. SOD activity is determined by following the rate of reduction of cytochrome *c* by superoxide anion, in a coupled system using xanthine and xanthine oxidase. No growth was observed on the plate in minimal agar after incubation with the test item, indicating its sterility. When not in use, the test item was stored in a dark, dry place at a temperature below 25 °C.

2.2. 90-Day repeated dose toxicity study

A 90-day oral subchronic toxicity study was conducted by the Centralized Service of Laboratory Animal Resources of the University of Córdoba (Córdoba, Spain). This study was performed under GLP and in compliance with the OECD Principles of Good Laboratory Practice (GLP), OECD Guideline No. 408 (1988), and the European Food Safety Authority (EFSA)'s "Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed." (EFSA, 2011). Animals were obtained from breeding colonies maintained according to Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Canadian Council of on Animal Care (CCAC) standards.

2.2.1. Animals and husbandry

Seven-week-old male and female Crl:OFA Sprague–Dawley (SD) rats (40/sex) were obtained from Charles River Laboratories S.L. (Lyon, France). Animals were acclimatized for 10 days, during which they were examined by a veterinarian and determined to be free of illness. At the start of dosing, at approximately 8 weeks of age, average body weight of the males was 206.5 g (range 172–226 g) and of the females

Table 3
Biochemistry results following 90 days of TetraSOD® administration. Data are presented as mean ± SD (n = 10/sex/group). The differences between control and male and female exposed rats were evaluated by K.W:Kruskal-Wallis test (K.W.) or by ANOVA test (F values). N.S. (Not significant) and the significance levels observed are * p < 0.05 versus sex-matched control.

CLINICAL BIOCHEMISTRY DATA SUMMARY

		MALE				FEMALE			
		Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
		(0 mg/kg/day)	(625 mg/kg/day)	(1667 mg/Kg/day)	(2500 mg/Kg/day)	(0 mg/kg/day)	(625 mg/kg/day)	(1667 mg/Kg/day)	(2500 mg/Kg/day)
		N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10
GLUC	MEAN	164.2	163.0	167.5	161.8	148.9	144.5	144.4	145.1
tmg/dL	ST. DEV.	21.1	36.7	50.6	45.9	37.4	38.8	43.0	46.0
		F(36.3) = 0.04 p = 0.99; N.S.				**F(36.3) = 0.02 p = 0.99; N.S.**			
UREA	MEAN	41.9	44.8	44.7	41.5	34.7	36.5	35.9	34.5
mg/dl	ST. DEV.	13.8	8.6	10.4	7.8	5.7	6.0	5.8	5.4
		KW = 1.41 p = 0.70; N.S.				**F(36.3) = 0.27 p = 0.84; N.S.**			
CREAT	MEAN	0.60	0.61	0.61	0.62	0.57	0.54	0.53	0.53
mg/dL	ST. DEV.	0.06	0.06	0.07	0.07	0.12	0.08	0.07	0.08
		F(36.3) = 0.91 p = 0.17; N.S.				**F(36.3) = 0.52 p = 0.66; N.S.**			
BILI-T	MEAN	0.7	0.6	0.7	0.6	0.9	0.9	0.7	0.8
mg/dL	ST. DEV.	0.3	0.2	0.3	0.3	0.5	0.3	0.4	0.4
		F(36.3) = 0.37 p = 0.77; N.S.				**KW = 3.84 p = 0.28; N.S.**			
Bile acids	MEAN	36.7	38.9	34.6	35.5	38.1	35.1	37.5	39.8
µMol	ST. DEV.	12.6	11.5	13.2	9.5	12.5	19.8	18.7	22.1
		F(36.3) = 0.25 p = 0.86; N.S.				**F(36.3) = 0.11 p = 0.95; N.S.**			
CHOL	MEAN	56.4	57.0	60.6	60.4	77.8	77.1	76.8	72.0
mg/dL	ST. DEV.	7.04	11.4	9.6	8.2	18.3	14.6	12.5	11.4
		F(36.3) = 0.57 p = 0.63; N.S.				**F(36.3) = 0.33 p = 0.79; N.S.**			
TRIGL	MEAN	116.8	126.8	114.7	116.1	94.9	90.8	90.4	97.0
mg/dL	ST. DEV.	26.2	40.7	34.2	33.0	50.4	31.20	12.4	58.5
		F(36.3) = 0.26 p = 0.85; N.S.				**KW) = 0.66 p = 0.88; N.S.**			
AST	MEAN	329.5	331.4	389.1	329.2	336.0	312.9	334.2	323.0
U/L	ST. DEV.	162.6	177.4	171.4	160.6	118.7	132.1	130.9	141.6
		F(36.3) = 0.31 p = 0.82; N.S.				**F(36.3) = 0.07 p = 0.98; N.S.**			
ALT	MEAN	294.5	252.3	282.9	254.4	284.8	247.3	205.1	277.4
U/L	ST. DEV.	154.5	88.9	101.2	90.5	168.4	102.0	115.6	134.7
		F(36.3) = 0.35 p = 0.79; N.S.				**F(36.3) = 0.75 p = 0.53; N.S.**			
ALKP	MEAN	161.7	185.0	153.7	145.3	116.1	123.7	125.5	115.8
U/L	ST. DEV.	48.8	46.9	37.4	42.4	37.7	41.3	46.8	35.7
		F(36.3) = 1.49 p = 0.23; N.S.				**KW = 1.01 p = 0.80; N.S.**			
GGT	MEAN	1.6	1.0	1.5	1.4	1.5	1.9	1.2	1.6
U/L	ST. DEV.	0.4	0.3	0.5	0.6	0.8	0.6	0.5	0.6
		F(36.3) = 3.03 p < 0.05; Significant				**KW = 5.2 p = 0.15; N.S.**			
ALB	MEAN	3.62	3.54	3.54	3.6	3.85	3.97	3.81	3.86
g/dl	ST. DEV.	0.16	0.16	0.10	0.2	0.19	0.30	0.21	0.21
		F(36.3) = 0.63 p = 0.59; N.S.				**F(36.3) = 0.85 p = 0.47; N.S.**			
TOT PROT	MEAN	6.1	6.0	6.1	6.0	6.2	6.4	6.1	6.2
g/dl	ST. DEV.	0.4	0.4	0.3	0.3	0.2	0.7	0.3	0.6
		F(36.3) = 0.33 p = 0.79; N.S.				**F(36.3) = 0.46 p = 0.86; N.S.**			
Na^+	MEAN	143.2	141.5	142.9	141.2	144.2	142.9	143.4	142.8
mmol/L	ST. DEV.	4.3	2.0	3.2	3.0	3.7	4.3	2.1	3.2
		F(36.3) = 0.98 p = 0.40; N.S.				**KW) = 0.70 p = 0.87; N.S.**			
K^+	MEAN	5.89	6.16	6.17	6.35	5.29	5.53	4.96	5.82
mmol/L	ST. DEV.	1.43	1.35	1.14	1.30	0.71	1.14	0.26	1.30
		F(36.3) = 0.020 p = 0.89; N.S.				**F(36.3) = 1.51 p = 0.22; N.S.**			
Ca	MEAN	10.14	10.09	10.00	10.05	9.98	9.84	9.97	9.85
mg/dL	ST. DEV.	0.69	0.36	0.31	0.46	0.21	0.29	0.14	0.35

(*continued on next page*)

Table 3 (*continued*)

CLINICAL BIOCHEMISTRY DATA SUMMARY									
		MALE				FEMALE			
		Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
		(0 mg/kg/day)	(625 mg/kg/day)	(1667 mg/Kg/day)	(2500 mg/Kg/day)	(0 mg/kg/day)	(625 mg/kg/day)	(1667 mg/Kg/day)	(2500 mg/Kg/day)
		N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10
		KW = 0.36 p = 0.94; N.S.				**F(36.3) = 0.92 p = 0.43; N.S.**			
PHOS	MEAN	6.25	6.59	6.62	6.94	4.91	5.27	5.20	5.66
mg/dL	ST. DEV.	1.75	1.82	1.17	1.42	0.55	1.06	0.61	1.57
		F(36.3) = 0.32 p = 0.80; N.S.				**F(36.3) = 0.88 p = 0.45; N.S.**			
Cl^-	MEAN	95.3	94.8	95.3	95.0	89.8	91.2	89.5	92.8
mmol/L	ST. DEV.	2.36	2.82	1.3	3.5	1.9	4.7	1.8	4.1
		KW = 0.48 p = 0.92; N.S.				**KW = 4.86 p = 0.18; N.S.**			
BUN	MEAN	17.46	18.67	18.62	17.29	14.46	15.21	14.76	14.37
mg/dL	ST. DEV.	5.74	3.57	4.32	3.27	2.39	2.51	2.44	2.26
		KW = 1.41 p = 0.70; N.S.				**F(36.3) = 0.24 p = 0.86; N.S.**			

GLUC: glucose; CREAT: creatinine; Bili-T: Bilirubin, total; CHOL: cholesterol, total; TRIGL: triglycerides; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALKP: alkaline phosphatase; GGT: Gamma-glutamyl transferase; ALB: albumin; TOT PROT: protein, total; Na^+:sodium; K^+:potassium; Ca: calcium; PHOS: phosphorus; BUN: blood urea nitrogen.
F: Statistics ANOVA test; K.W:Kruskal-WallisStatistic; N.S.: Not Significant.
*: One-Way Analysis of Variance (ANOVA) and Bonferroni/Dunn's Multiple Comparison Test. *when $p < 0.05$.

Table 4
Microscopic findings for rats receiving vehicle control or 2500 mg/kg TetraSOD® for 90 days. N = 10/sex/group. No significant differences between groups were observed for either sex.

Organ	Finding	Males		Females	
		Control	2500 mg/kg/d	Control	2500 mg/kg/d
Adrenals	Vacuolation: zone fasciculata cell	1	0	2	1
	Vacuolation: zone glomerular cell	1	0	0	0
Epididymis	Edema	1	0	–	–
Heart	Edema	1	1	1	0
Kidneys	Tubular basophilia	3	0	1	1
	Congestion	1	0	0	0
	Cellular infiltrate	0	0	1	1
	Focal hydronephrosis in tubular cells	1	1	1	0
	Focal atrophy in tubular cells	0	0	0	1
	Tubular mineralization	0	1	0	0
Liver	Vacuolation, hepatocellular	2	4	1	0
	Glycogen deposition	2	2	0	1
Lungs	Congestion	0	1	1	0
	Alveolar macrophages	0	0	0	1
	Edema	1	2	0	2
Mandibular lymph nodes	Congestion	0	0	0	1
	Lymphoid hyperplasia	2	2	2	3
Mesenteric lymph nodes	Congestion	0	0	1	0
	Lymphoid hyperplasia	2	3	4	3
Pituitary gland	Cyst(s)	0	0	1	0
Spleen	Hemopoiesis	5	6	6	6
	Hemosiderin	2	3	2	2
Testes	Edema	0	1	–	–
Thymus	Congestion	2	1	0	0
Trachea	Mucosal gland hyperplasia	1	0	0	1

was 178.3 g (153–198 g).

Rats were individually housed in cages type 3H with Souralit 29/12 Plus (Souralit, S.L., Gerona, Spain) aspen wood bedding, maintained in a room with optimum hygienic conditions behind a barrier system, supplied with an air-conditioned with a minimum of 10–15 air changes per hour and continuously monitored environment with target ranges for temperature of 21 ± 2 °C and for a relative humidity between 30 and 70%, under a 12 h light/dark cycle. Cage card contained information of study code, sex, dose group, and individual animal identification. Food was available without restriction, using standard dry pelleted diet for rodents Scientific, Animal Food &Engineering (SAFE) A04 rat maintenance diet (Panlab, S.L.U., Cornella de Llobregat, Barcelona, Spain). Community tap water (EMACSA, Cordoba Water company, Córdoba, Spain), filtered and autoclaved was available *ad libitum*.

2.2.2. Doses selection and administration

For the 90-day study, dietary dose individual formulations were

Table 5
Absolute organ weight of male and female rats fed with TetraSOD® for 90 days. Data are presented as mean ± standard deviation (n = 10/sex/group). The differences between control and exposed groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA (F values).

ORGAN WEIGHT DATA SUMMARY

SEX: MALE						SEX: FEMALE					
Dose of TetraSOD		Group 1 (0 mg/kg/day) N = 10	Group 2 (625 mg/kg/day) N = 10	Group 3 (1667 mg/kg/day) N = 10	Group 4 (2500 mg/kg/day) N = 10			Group 1 (0 mg/kg/day) N = 10	Group 2 (25 mg/kg/day) N = 10	Group 3 (100 mg/kg/day) N = 10	Group 4 (400 mg/kg/day) N = 10
BODY W. (g)	**MEAN**	510.60	510.00	523.80	510.10	**BODY W. (g)**	**MEAN**	299.70	292.60	294.30	282.40
	ST. DEV.	38.04	38.15	55.25	46.65		**ST. DEV.**	24.12	35.48	27.96	21.35
F(36,3) = 0.22 p = 0.87; N.S.						**F(36,3) = 0.67 p = 0.57; N.S.**					
BRAIN (g)	**MEAN**	2.07	2.18	2.05	2.06	**BRAIN (g)**	**MEAN**	1.90	1.84	1.91	1.81
	ST. DEV.	0.10	0.51	0.09	0.12		**ST. DEV.**	0.09	0.14	0.14	0.29
KW = 0.28 p = 0.96; N.S.						**F(36,3) = 0.72 p = 0.54; N.S.**					
LIVER (g)	**MEAN**	14.86	14.24	15.13	14.40	**LIVER (g)**	**MEAN**	8.08	7.90	7.79	7.12
	ST. DEV.	1.75	1.40	2.43	2.36		**ST. DEV.**	0.63	1.25	0.73	0.98
F(36,3) = 0.41 p = 0.74; N.S.						**KW = 6.74 p = 0.08; N.S.**					
HEART (g)	**MEAN**	1.60	1.71	1.63	1.62	**HEART (g)**	**MEAN**	1.05	1.11*	1.06	0.99
	ST. DEV.	0.25	0.14	0.19	0.36		**ST. DEV.**	0.07	0.09	0.08	0.07
F(36,3) = 0.39 p = 0.75; N.S.						**KW = 9.49 * p < 0.05, Significant differences between group 2 and 4**					
SPLEEN (g)	**MEAN**	0.74	0.79	0.86	0.76	**SPLEEN (g)**	**MEAN**	0.54	0.51	0.55	0.52
	ST. DEV.	0.11	0.08	0.21	0.08		**ST. DEV.**	0.06	0.05	0.07	0.06
KW = 1.57 p = 0.66; N.S.						**KW = 1.76 p = 0.62; N.S.**					
KIDNEYS (g)	**MEAN**	3.37	3.45	3.54	3.42	**KIDNEYS (g)**	**MEAN**	2.13	2.05	2.10	1.97
	ST. DEV.	0.37	0.61	0.50	0.52		**ST. DEV.**	0.19	0.21	0.21	0.26
KW = 0.56 p = 0.90; N.S.						**F(36,3) = 1.03 p = 0.38; N.S.**					
THYMUS (g)	**MEAN**	0.46	0.46	0.42	0.41	**THYMUS (g)**	**MEAN**	0.31	0.33	0.31	0.31
	ST. DEV.	0.09	0.09	0.12	0.15		**ST. DEV.**	0.08	0.09	0.05	0.06
F(36,3) = 0.59 p = 0.62; N.S.						**F(36,3) = 0.17 p = 0.90; N.S.**					
TESTES (g)	**MEAN**	3.92	3.79	4.04	3.88	**UTERUX/CERVIX (g)**	**MEAN**	0.67	0.70	0.76	0.72
	ST. DEV.	0.19	0.23	0.32	0.31		**ST. DEV.**	0.10	0.10	0.12	0.14
F(36,3) = 1.53 p = 0.22; N.S.						**F(36,3) = 0.95 p = 0.42; N.S.**					
EPIDIDIMS (g)	**MEAN**	1.95	2.25	2.04	2.07	**OVARIES (g)**	**MEAN**	0.258	0.285	0.235	0.209
	ST. DEV.	0.36	0.37	0.89	0.55		**ST. DEV.**	0.083	0.065	0.061	0.078
F(36,3) = 0.45 p = 0.71; N.S.						**F(36,3) = 2.01 p = 0.12; N.S.**					
ADRENALS (g)	**MEAN**	0.064	0.067	0.064	0.061	**ADRENALS (g)**	**MEAN**	0.065	0.080	0.088	0.075
	ST. DEV.	0.038	0.018	0.039	0.049		**ST. DEV.**	0.019	0.031	0.034	0.010
KW = 2.13 p = 54; N.S.						**KW = 1.43 p = 0.31; N.S.**					

N.S.: Not significant.

prepared daily for the whole experiment (13 weeks), and on Friday, they were also prepared for the weekend. For dietary dose individual formulation, the test substance was included in 3 mL neutral gelatine (from a solution prepared with12 slides/100 mL warm water). The quantities of the test substance added (μg of TetraSOD ®) depended on the dose selected for each experimental group, and were solidified at 4 °C, and stored at this temperature overnight, protected from light. Homogeneity of the dietary dose formulations and their stability were confirmed to be at least 5 days. Rats (10/sex/group) were orally administered 0 (control), 625, 1667, or 2500 mg/kg/day TetraSOD ®). Pork gelatine was used as the vehicle for the test item and also administered as the vehicle control to untreated animals.

The dose levels were set based on the results of a 14-day oral palatability study performed with the test item at the same laboratory, data regarding previous toxicological studies (median lethal dose LD_{50} > 2500 mg/kg, following the OECD test guideline 423, 2001) (AESAN, 2013) and potential human consumption (calculated accumulated intake of 800 mg/day, AECOSAN, 2017). 15 females eight-week-old Crl:OFA Sprague–Dawley (SD) rats obtained from the same provider were used for the palatability study. There were randomly distributed in 3 groups (n = 5), and dosed 0 (control, vehicle: gelatine), 1667, and 2500 mg/kg/day TetraSOD®. After 14 days the animals were sacrificed and necropsied. Animals did not show any visible signs of rejection of the vehiculated test item, neither any adverse effect.

2.2.3. Clinical observations. Body weight, food and water consumption

Animals were monitored twice daily for mortality and overt signs of toxicity and detailed clinical examinations were performed weekly. The observations were carried out by removing the animals from their cages at the same time every day. Body weights, food intake, and water consumption were recorded weekly. The mean body weights per group and sex were calculated weekly from the individual animals.

Ophthalmological examinations (cornea, crystalline lens, conjunctivae, sclera, iris and fundus) were performed prior to treatment and prior to euthanasia.

2.2.4. Haematology and blood chemistry

Blood was collected from the heart (intracardiac injection) at week

Table 6
Relative weight/body weight of male and female rats fed with different doses of TetraSOD® for 90 days. Vales are presented as mean ± SD (n = 10/sex/group). The differences between control and exposed groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA (F values).

ORGAN WEIGHT/BODY WEIGHT RATIO DATA SUMMARY

SEX: MALE						SEX: FEMALE					
Dose of TetraSOD		Group 1 (0 mg/kg/day)	Group 2 (625 mg/kg/day)	Group 3 (1667 mg/kg/day)	Group 4 (2500 mg/kg/day)			Group 1 (0 mg/kg/day)	Group 2 (625 mg/kg/day)	Group 3 (1667 mg/kg/day)	Group 4 (2500 mg/kg/day)
		N = 10	N = 10	N = 10	N = 10			N = 10	N = 10	N = 10	N = 10
BRAIN	**MEAN**	0.408	0.430	0.395	0.406	**BRAIN**	**MEAN**	0.640	0.637	0.656	0.646
(%)	**ST. DEV.**	0.037	0.113	0.041	0.032	(%)	**ST. DEV.**	0.076	0.083	0.085	0.120
KW = 0.67 p = 0.87; N.S.						**KW = 1.10 p = 0.77; N.S.**					
LIVER	**MEAN**	2.909	2.793	2.877	2.814	**LIVER**	**MEAN**	2.709	2.704	2.651	2.521
(%)	**ST. DEV.**	0.237	0.221	0.211	0.275	(%)	**ST. DEV.**	0.260	0.312	0.118	0.303
F(36,3) = 0.51 p = 0.67; N.S.						**F(36,3) = 1.12 p = 0.35; N.S.**					
HEART	**MEAN**	0.313	0.337	0.312	0.315	**HEART**	**MEAN**	0.353	0.382	0.360	0.351
(%)	**ST. DEV.**	0.031	0.034	0.036	0.053	(%)	**ST. DEV.**	0.030	0.024	0.032	0.022
KW = 3.29 p = 0.34; N.S.						**F(36,3) = 2.72 p = 0.06; N.S.**					
SPLEEN	**MEAN**	0.144	0.156	0.165	0.150	**SPLEEN**	**MEAN**	0.181	0.175	0.187	0.183
(%)	**ST. DEV.**	0.016	0.022	0.042	0.013	(%)	**ST. DEV.**	0.017	0.018	0.026	0.016
KW = 1.68 p = 0.64; N.S.						**F(36,3) = 0.11 p = 0.95; N.S.**					
KIDNEYS	**MEAN**	0.660	0.676	0.674	0.670	**KIDNEYS**	**MEAN**	0.713	0.707	0.719	0.699
(%)	**ST. DEV.**	0.055	0.116	0.049	0.070	(%)	**ST. DEV.**	0.043	0.077	0.094	0.092
F(36,3) = 0.09 p = 0.96; N.S.						**F(36.3) = 0.6992 p = 0.5587; N.S.**					
THYMUS	**MEAN**	0.090	0.091	0.080	0.079	**THYMUS**	**MEAN**	0.104	0.113	0.106	0.110
(%)	**ST. DEV.**	0.019	0.014	0.020	0.026	(%)	**ST. DEV.**	0.026	0.034	0.021	0.015
F(36,3) = 0.98 p = 0.41; N.S.						**F(36,3) = 0.29 p = 0.82; N.S.**					
TESTES	**MEAN**	0.772	0.747	0.780	0.765	**UTE./CERV.**	**MEAN**	0.226	0.241	0.260	0.257
(%)	**ST. DEV.**	0.066	0.075	0.109	0.080	(%)	**ST. DEV.**	0.042	0.038	0.052	0.049
F(36,3) = 0.28 p = 0.83; N.S.						**F(36,3) = 1.21 p = 31; N.S.**					
EPIDIDIMS	**MEAN**	0.385	0.442	0.396	0.412	**OVARIES**	**MEAN**	0.0863	0.0989	0.0790	0.0737
(%)	**ST. DEV.**	0.085	0.078	0.183	0.128	(%)	**ST. DEV.**	0.0289	0.0270	0.0143	0.0269
F(36,3) = 0.38 p = 0.76; N.S.						**F(36,3) = 1.91 p = 0.14; N.S.**					
ADRENALS	**MEAN**	0.0124	0.0131	0.0122	0.0119	**ADRENALS**	**MEAN**	0.0219	0.0275	0.0297	0.0265
(%)	**ST. DEV.**	0.0078	0.0034	0.0073	0.0098	(%)	**ST. DEV.**	0.0063	0.0113	0.0101	0.0029
KW = 3.07 p = 0.37; N.S.						**F(36,3) = 1.56 p = 0.21; N.S.**					

N.S.: Not Significant.

13, prior to necropsy, for haematology, coagulation, and clinical chemistry evaluations. Haematological parameters were evaluated on an automatic haematology analyser Cell-Dyn 3700 (Abbot, GMI, MI, USA) and included: erythrocyte count (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet (thrombocyte) count (PLT), and total leukocyte count (WBC) with differential (reticulocytes (RE), neutrophils (NE), lymphocytes (LY), monocytes (MO), basophils (BA), and eosinophils (EO)). Coagulation parameters evaluated were prothrombin time (PT) and activated partial thromboplastin time (APTT).

Plasma clinical chemistry parameters were analysed with an automatic chemistry analyser Cobas 6000 (Roche Diagnostics, IN, USA), and included glucose (GLC), total protein (PRO), albumin (ALB), triglycerides (TG), total cholesterol (TC), bile acids, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), total bilirubin (BIL), urea, blood urea nitrogen (BUN), creatinine (CRE), sodium (Na), potassium (K), and chloride (Cl), calcium (Ca), and inorganic phosphorous (Pi) levels.

2.2.5. Necropsy and organ weights

Following blood collection, animals were euthanized by exsanguination and CO_2 under deep anaesthesia and subjected to necropsy and gross pathological examination. The organs that were collected, weighed, and preserved included the adrenal glands, aorta, bone marrow (femur), brain (medulla/pons, cerebellum, cerebrum), epididymis, oesophagus, eyes with optic nerve, heart, kidneys, large intestine (cecum, colon, rectum), larynx, liver, lungs (with main bronchi and bronchioles), mandibular and mesenteric lymph nodes, mammary gland, ovaries, pancreas, pituitary gland, prostate with seminal vesicles, salivary glands (mandibular), sciatic nerve, skeletal muscle (thigh), skin (abdominal), small intestine (duodenum, jejunum, ileum), spinal cord (cervical, mid-thoracic, lumbar), spleen, stomach (glandular and non-glandular), testes, thymus, thyroid and parathyroid, tongue, trachea, urinary bladder, uterus (with cervix and oviducts), and all gross lesions or other organ or tissue with macroscopic alterations. Tissues were fixed in 10% neutral phosphate buffered formalin. The preserved tissues were processed and embedded in paraffin, sectioned, and stained with haematoxylin and eosin. Adrenal glands, brain, epididymis, heart, kidneys, testes, thymus, ovaries, spleen, and uterus were weighed after the removal of adhering fat and tissues, with paired organs weighed together. Histopathology was performed on all preserved organs and

Table 7
Relative weight/brain weight of male and female rats fed with different doses of TetraSOD® for 90 days. Vales are presented as mean ± SD (n = 10/sex/group). The differences between control and exposed groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA (F values).

ORGAN WEIGHT/BRAIN WEIGHT RATIO DATA SUMMARY

SEX: MALE						SEX: FEMALE					
Dose of TetraSOD		Group 1 (0 mg/kg/day)	Group 2 (625 mg/kg/day)	Group 3 (1687 mg/kg/day)	Group 4 (2500 mg/kg/day)			Group 1 (0 mg/kg/day)	Group 2 (625 mg/kg/day)	Group 3 (1667 mg/kg/day)	Group 4 (2500 mg/kg/day)
		N = 10	N = 10	N = 10	N = 10			N = 10	N = 10	N = 10	N = 10
BODY WEIGHT (%)	**MEAN**	24709.7	24224.8	25558.3	24788.0	**BODY WEIGHT (%)**	**MEAN**	15833.2	15943.8	15459.8	16163.5
	ST. DEV.	2313.0	4279.9	2630.0	1926.3		**ST. DEV.**	1956.1	2101.2	1895.8	4132.5
F(36,3) = 0.35 p = 0.78; N.S.						**KW = 1.03 p = 0.79; N.S.**					
LIVER (%)	**MEAN**	720.60	678.92	739.14	698.94	**LIVER (%)**	**MEAN**	426.21	431.42	409.84	403.93
	ST. DEV.	104.80	144.38	120.42	99.71		**ST. DEV.**	42.27	79.23	55.16	95.59
F(36,3) = 0.48 p = 0.69; N.S.						**KW = 2.63 p = 0.45; N.S.**					
HEART (%)	**MEAN**	77.74	81.14	79.38	78.27	**HEART (%)**	**MEAN**	55.57	60.64	55.39	56.86
	ST. DEV.	13.72	14.83	9.86	15.05		**ST. DEV.**	5.06	6.06	5.81	16.01
F(36,3) = 0.12 p = 0.94; N.S.						**KW = 5.88 p = 0.11; N.S.**					
SPLEEN (%)	**MEAN**	35.76	37.61	41.91	37.03	**SPLEEN (%)**	**MEAN**	28.66	27.68	28.74	29.52
	ST. DEV.	5.98	8.09	10.19	3.43		**ST. DEV.**	4.27	3.16	4.36	6.88
KW = 2.07 p = 0.55; N.S.						**F(36,3) = 0.24 p = 0.86; N.S.**					
KIDNEYS (%)	**MEAN**	163.00	166.12	172.52	166.08	**KIDNEYS (%)**	**MEAN**	112.75	111.96	110.73	113.80
	ST. DEV.	20.47	42.35	23.76	21.40		**ST. DEV.**	14.62	14.09	17.96	38.72
F(36,3) = 0.19 p = 0.89; N.S.						**KW = 1.17 p = 0.75; N.S.**					
THYMUS (%)	**MEAN**	22.17	22.14	20.52	19.68	**THYMUS (%)**	**MEAN**	16.28	17.97	16.16	18.03
	ST. DEV.	4.70	5.45	5.54	6.94		**ST. DEV.**	3.99	5.34	2.85	6.42
F(36,3) = 0.46 p = 0.70; N.S.						**KW = 1.10 p = 0.77; N.S.**					
TESTES (%)	**MEAN**	189.84	180.45	197.14	188.81	**UTE./CERV. (%)**	**MEAN**	35.37	38.17	39.82	40.76
	ST. DEV.	12.61	35.32	16.02	17.11		**ST. DEV.**	5.00	6.08	7.93	8.98
F(36,3) = 0.95 p = 0.42; N.S.						**KW = 2.88 p = 0.40; N.S.**					
EPIDIDIMS (%)	**MEAN**	94.39	107.73	99.60	100.43	**OVARIES (%)**	**MEAN**	13.66	15.55	12.32	12.25
	ST. DEV.	18.00	30.56	43.58	26.69		**ST. DEV.**	4.57	3.64	3.17	5.99
KW = 1.37 p = 0.71; N.S.						**F(36,3) = 1.19 p = 0.32; N.S.**					
ADRENALS (%)	**MEAN**	3.05	3.24	3.13	2.97	**ADRENALS (%)**	**MEAN**	3.44	4.35	4.68	4.30
	ST. DEV.	1.74	1.26	1.88	2.43		**ST. DEV.**	1.03	1.73	1.99	1.31
KW = 1.63 p = 0.65; N.S.						**KW = 2.93 p = 0.40; N.S.**					

N.S.: Not Significant.

tissues of the vehicle control and TetraSOD® high dose group only, unless adverse findings were observed.

2.3. Statistical analysis

Statistical analysis was conducted for body weight, food and water consumption, haematology, clinical chemistry, organ weights, and organ weight ratios using Stata version 12, SPSS version 18, and GraphPad Instant. Analysis of body weight, food, and water consumption was conducted using a two-factor, repeated-measures analysis of variance (RM-ANOVA). Homogeneity of variances was tested using Bartlett's test. Interactions between doses and other variables were included in the models. Other continuous variables were analysed using one-way ANOVA. Normality was tested using Kolmogorov-Smirnov's test. Data displaying non-normality were compared via Kruskal-Wallis's test followed by Bonferroni/Dunn's Multiple Comparisons Tests. For necropsy and histological findings, differences were tested using the chi-square test. Males and females were considered separately for each analysis. P-values were two-tailed and an alpha of 0.05 was used to assess statistical significance.

3. Results

A 90-day oral subchronic toxicity study was conducted in male and female Sprague–Dawley rats using doses of 0 (control, gelatine), 625, 1667, and 2500 mg/kg/day TetraSOD®. No mortality or test article related clinical signs were observed in animals of either sex in any group during the study period. One male receiving 625 mg/kg/day TetraSOD® displayed a light periocular dermatitis in the right eye in week 2, but this resolved by the week 3 evaluation and was considered non-treatment related.

Overall, TetraSOD® administration resulted in no differences in mean body weight for animals of either sex compared to control animals (Fig. 1). Week-to-week body weight gain was not significantly different for male animals receiving TetraSOD® versus the vehicle control (Fig. 2). Female rats in the treated groups had slightly lower weekly body weight gain compared to the control group, but none of these differences were statistically significant (Fig. 2).

Food and water consumption data per week along the study of both male and female animals in the treatment groups were not different than that of the control group (Figs. 3 and 4). A significant difference was determined by ANOVA for water consumption in females during

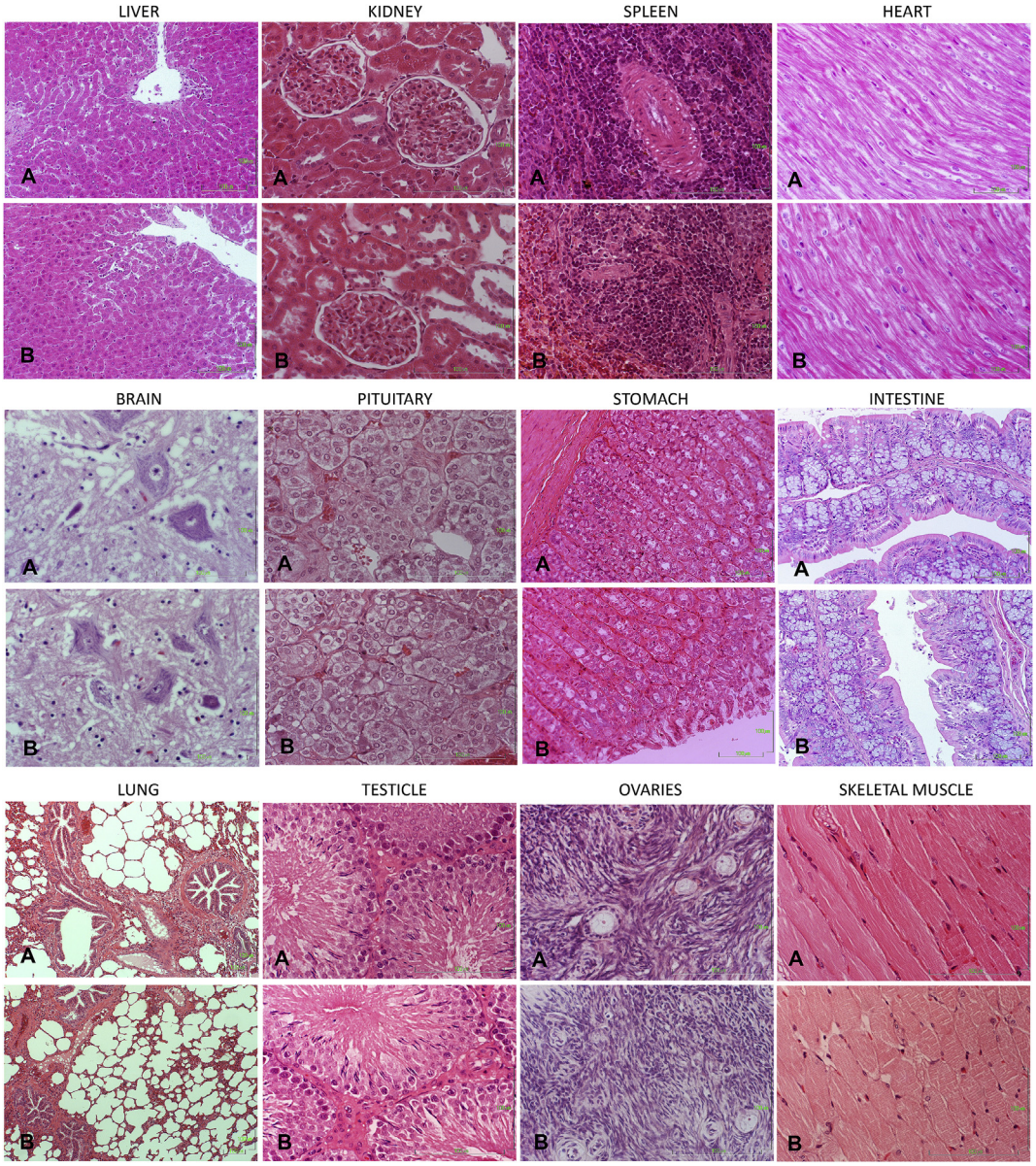

Fig. 5. Histopathological findings in liver, kidney, spleen, heart, brain, pituitary, stomach, intestines, lung, testicles and ovaries, and skeletal muscle of male and female untreated rats (control, A) and male and female rats exposed to the highest dose of TetraSOD® (2500 mg/kg/day) for 90 days. Representative sections of these organs (50 mm). In liver, normal hepatic cords and normal polyhedral hepatocytes appeared with central nucleus and clear cytoplasm in all cases. In the kidney, renal parenchyma with normal glomeruli and renal tubules in male and female control rats (A), which are conserved in treated rats with TetraSOD® (B) are shown. Detail of the spleen parenchyma apparently normal in control and treated rats (A, B). In heart, normal cardiac fibres were observed in all cases (A, B). In brain, motor neurons of cerebral cortex were normal in all cases (A, B). Detail of apparently normal pituitary were observed in control and treated rats (A, B). Detail of the glandular stomach with mucosal and glandular cells apparently normal in control and treated rats (A, B). Intestinal villi with abundant apparently normal enterocytes were observed in all cases. Detail of control bronchial epithelia, without any alterations in bronchia and alveoli in untreated and exposed rats (A, B). Testicles of male rats showed normal seminiferous tubules and interstitial space (A) which were maintained in treated male rats (B). Ovaries from control and treated female rats (A, B) showed normal follicles in all cases. Detail of the normal striated skeletal muscle from control and treated female rats were shown (A, B).

week 11 (with animals in the low dose TetraSOD® drinking more than the other groups) but use of Bonferroni's multiple comparison test did not demonstrate statistical significance. Thus, TetraSOD® administration did not have any effects on food or water consumption.

No changes were observed in ophthalmology parameters for animals of any group during the study.

Haematology and blood coagulation measurements performed at the end of the study revealed no statistically significant differences between animals administered TetraSOD® and those receiving vehicle control (Table 1). All values were within historical/expected ranges.

Total and differential leukocyte counts performed at the end of the study revealed no statistically significant differences between animals administered TetraSOD® and those receiving vehicle control (Table 2). All values were within historical/expected ranges.

Blood biochemistry measurements taken at the end of the study are shown in Table 3. The only statistically significant difference occurred in GGT for male animals, with the 625 mg/kg/day TetraSOD® group showing a significant reduction compared to control. Since this only occurred at the lowest dose of TetraSOD® and was only observed in male animals, this effect was deemed not treatment-related.

Upon necropsy, organs and tissues from animals in the control and 2500 mg/kg/day TetraSOD® groups were examined macroscopically and microscopically. Macroscopic findings were minimal and included reddish discoloration in the small intestine (jejunum), kidneys with

pelvic dilation, and alopecia. These are considered normal background findings in rats of this age and strain. No statistically significant differences were found in the occurrence of these findings between groups.

Microscopic findings are detailed in Table 4. These findings were considered normal background findings in this strain, and no statistically significant differences in their occurrence were found between groups. Thus, findings were considered not treatment-related.

Terminal body weight and absolute organ weights are detailed in Table 5. No statistically significant differences in absolute organ weights were noted in any treatment group compared to control. No significant differences between groups were observed in the relative organ weight/body weight (Table 6) or organ to brain weight ratio in animals of either sex (Table 7).

The histological findings described in liver, kidney, spleen, heart, brain, pituitary, stomach, intestines, lung, testicles and ovaries, and skeletal muscle of male and female treated rats up to a concentration of 2500 mg/kg/day TetraSOD® were similar to those observed in the untreated groups in both sexes control groups (Fig. 5).

4. Discussion

Due to their high nutrient content, microalgae have been widely used in aquaculture as a complete food source or as a food additive (Muller-Feuga, 2000). In fact, it is estimated that 30% of worldwide algal production is sold for use in animal feed (Becker, 2003). Algae used in aquaculture must meet several requirements including lack of toxicity, cell size appropriate for ingestion, ease of culture (rapid growth, resistance to variable culture conditions), and a favourable nutrient composition (Lavens and Sorgeloos, 1996; Hallmann, 2007). *Tetraselmis* sp. including *T. chuii* are among the most frequently species used for aquaculture due to meeting these criteria (Lavens and Sorgeloos, 1996; Spolaore et al., 2006; Hallmann, 2007; Becker, 2003; Muller-Feuga et al., 2007). Due to these favourable characteristics, microalgae as *T. chuii* have great potential as a sustainable and nutrient-dense food source for humans (Spolaore et al., 2006; Borowitzka, 2013).

In contrast to other microalgae with potential for use in human food (i.e., *Chlorella,* spirulina), very little has been published regarding the safety of *T. chuii*. Despite its long history of beneficial use in aquaculture, no publicly available toxicology studies of *T. chuii* were available.

The Regulations of novel foods (Reg CE 258/97; Reg EU 2015/2283), and their supporting documents (97/618/EC: Commission Recommendation and the EFSA guidance for the preparation of applications for authorization of a novel food (Turck et al., 2016)), state that a novel food under no circumstance can be a risk for the consumer. In order to guarantee the safety of the novel food a subchronic toxicity study is indicated, in accordance with international guidelines (e.g. OECD) and according to the principles of GLP. In this sense, the 90-day study of OECD guideline 408 (1998, 2018) provides information on the possible health hazards likely to arise from repeated exposure over a prolonged period of time covering post-weaning maturation and growth into adulthood of the test animals (Mellado-García et al., 2016; Llana-Ruiz-Cabello et al., 2017). The study will provide information on the major toxic effects, indicate target organs and the possibility of accumulation of test chemical, and can provide an estimate of a no-observed-adverse-effect level (NOAEL) of exposure which can be used in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

To address this issue, the safety of the commercial product TetraSOD®, composed of 100% lyophilized *T. chuii strain CCFM3,* was evaluated in a 90-day subchronic toxicology study in rats. Rats administered doses up to 2500 mg/kg/day TetraSOD® exhibited no adverse effects on any tested parameter including clinical signs, body weights, haematology, blood chemistry, absolute organ weights and relative organ weight/body weight or organ weight/brain weight in comparison to control rats. These results are in agreement with the negative pathological changes detected in the histopathological analysis. Therefore, the NOAEL for this study was greater than the highest tested dose of 2500 mg/kg/day. Together, these findings support the safe use of TetraSOD® in the human diet.

Several subchronic studies performed with other microalgae supplements are available in the scientific literature, and similarly to the results obtained in this work, the NOAEL reported are greater than the highest dose assayed, because globally, a lack of adverse effects in male and female rats in the 90-d dietary studies were found. Thus, the safety of Whole Algalin Protein (WAP) from dried milled *Chlorella protothecoides* was evaluated by a subchronic study in rats consuming feed, and the NOAEL was equivalent to 4800–5500 mg/kg/day, and although several endpoints exhibited significant effects, they were considered not relevant and not dose-related (Szabo et al., 2013). Similarly, the NOAEL for the green algae *Chlorella sorokiniana*, strain CK-22, administered to rats through the diet, in a 13- week subchronic toxicity assay was estimated to be between 5940 mg/kg/day for males and 6410 mg/kg/day for females. This study was carried out following basically the OECD Guideline 408, but did not conduct a full histopathological examination (Himuro et al., 2017). In the case of a dried algal biomass derived from *Klebsormidium flaccidum* var ZIVO, its safety as an ingredient in foods was evaluated by a subchronic dietary toxicity study in rats, and the NOAEL were greater than the highest dose tested (7900–9700 mg/kg/day) (Brickel et al., 2018). The absence of toxicity seen when this novel microalgal lyophilized biomass was assayed, is consistent with the evaluation of other microalgal-derived substances. As an illustration: structuring fat produced using a heterotrophic fermentation process by a strain of *Prototeca moriformis* (Matulka et al., 2016), a docosahexaenoic acid-rich oil derived from *Schizochytrium sp.* (Schmitt et al., 2012), high oleic acid-containing oil produced from a genetically engineered strain of *P. moriformis* (Szabo et al., 2014), or a high EPA-containing ingredient from *Nannochloropsis oculata* at up to 2000 mg/kg/day (Kagan et al., 2014). All these studies demonstrated the safety of these food ingredients or novel food derived from microalgae.

Considering the NOAEL provided in this study, greater than the highest dose of 2500 mg/kg/day assayed, it is interesting to make a comparison with the real exposure scenario to consumers. Considering an accumulated intake of 800 mg/day (this is the highest content of *T. chuii* in all the food products where it has been authorised), equivalent to 11.4 mg/kg/day for an adult consumer weighing 70 kg, the NOAEL found in this study was 200-fold higher than this maximal potential exposure. Hence, no likely subchronic effects associated to the consumption of TetraSOD® could be expected, under the experimental conditions assayed.

5. Conclusion

The present work reported for the first time that after a 90-day repeated dose oral exposure to TetraSOD® up to 2500 mg/kg/day, male and female rats did not show any change on body weight, food and water consumption, as well as any effect on haematological and biochemical parameters evaluated. Moreover, after necropsy, no significant differences between treated and control rats for terminal body weight or any absolute or relative weighted organs, and no histopathological changes were detected during the treatment with TetraSOD®. Consequently, the NOAEL obtained in the present study for TetraSOD® is established greater than 2500 mg/kg/day under the test conditions followed, and no subchronic toxic effects are likely to occur.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This study was sponsored by Fitoplancton Marino, SL., the manufacturer of TetraSOD®.

References

AECOSAN, 2017. Informe del Comité Científico de la Agencia Española de Consumo, Seguridad Alimentaria y Nutrición (AECOSAN) en relación a una solicitud de evaluación inicial para la comercialización de un liofilizado de la microalga marina *Tetraselmis chuii* en complementos alimenticios en el marco del Reglamento (CE) Nº 258/97 sobre nuevos alimentos y nuevos ingredientes alimentarios. Revista del Comité Científico de la AECOSAN 25, 11–21.

AESAN, 2013. Informe del Comité Científico de la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) en relación a una solicitud de evaluación inicial para la comercialización de la microalga marina *Tetraselmis chuii* en el marco de Reglamento (CE) Nº 258/97 sobre nuevos alimentos y nuevos ingredientes alimentarios. Revista del Comité Científico de la AECOSAN 18, 11–27.

Banskota, A.H., Sperker, S., Stefanova, R., McGinn, P.J., O'Leary, S.J.B., 2019. Antioxidant properties and lipid composition of selected microalgae. J. Appl. Phycol. 31, 309–318. https://doi.org/10.1007/s10811-018-1523-1.

Barka, A., Blecker, C., 2016. Microalgae as a potential source of single-cell proteins. A review. Biotechnol. Agron. Soc. Environ. 20, 427–436.

Becker, W., 2003. Microalgae in human and animal nutrition. In: Handbook of Microalgal Culture: Biotechnology and Applied Phycology, (Chapter 18). https://doi.org/10.1002/9780470995280.ch18.

Becker, E.W., 2007. Micro-algae as a source of protein. Biotechnol. Adv. 25, 207–210. https://doi.org/10.1016/j.biotechadv.2006.11.002.

Bonilla-Ahumada, F. de J., Khandual, S., Lugo-Cervantes, E. del C., 2018. Microencapsulation of algal biomass (*Tetraselmis chuii*) by spray-drying using different encapsulation materials for better preservation of beta-carotene and antioxidant compounds. Algal Res 36, 229–238. https://doi.org/10.1016/j.algal.2018.10.006.

Borowitzka, M.A., 2013. High-value products from microalgae-their development and commercialisation. J. Appl. Phycol. 25, 743–756. https://doi.org/10.1007/s10811-013-9983-9.

Brickel, J.A., Matulka, R.A., Steffek, A.E., 2018. KALGAE™ (*Klebsormidium flaccidum* var. ZIVO) dried algal biomass - 90-day dietary toxicity study and genotoxicity studies. Toxicol. Rep. 5, 959–969. https://doi.org/10.1016/j.toxrep.2018.09.002.

Brown, M.R., Mular, M., Miller, I., Farmer, C., Trenerry, C., 1999. The vitamin content of microalgae used in aquaculture. J. Appl. Phycol. 11, 247–255. https://doi.org/10.1023/A:1008075903578.

Brown, M.R., Jeffrey, S.W., 1992. Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. Amino acids, sugars and pigments. J. Exp. Mar. Biol. Ecol. 161, 91–113. https://doi.org/10.1016/0022-0981(92)90192-D.

Butcher, R.W., 1959. An Introductory Account of the Smaller Algae of British Coastal Waters: Part I: Introduction and Chlorophyceae. Great Britain: Ministry of Agriculture, Fisheries and Food, Fish Investigation IV (Part 1). H.M. Stationery Office, pp. 74.

Canada Food and Drug Regulation (C.R.C., c870), 2019. Section B28.002. Current to June 6. https://laws-lois.justice.gc.ca/PDF/C.R.C.,_c._870.pdf, Accessed date: 11 July 2019.

Chacón-Lee, T.L., González-Mariño, G.E., 2010. Microalgae for "healthy" foods-possibilities and challenges. Compr. Rev. Food Sci. Food Saf. 9, 655–675. https://doi.org/10.1111/j.1541-4337.2010.00132.x.

Commission Implementing Regulation (EU) 2017/2470 of 20, December 2017. Establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32017R2470.

Commission Recommendation of 29 July 1997 (97/618/EC) concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients and the preparation of initial assessment reports under Regulation (EC) No 258/97 of the European Parliament and of the Council. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A31997H0618.

Dunstan, G.A., Volkman, J.K., Jeffrey, S.W., Barrett, S.M., 1992. Biochemical composition of microalgae from the green algal classes *Chlorophyceae* and *Prasinophyceae*. 2. Lipid classes and fatty acids. J. Exp. Mar. Biol. Ecol. 161, 115–134. https://doi.org/10.1016/0022-0981(92)90193-E.

EFSA, 2011. Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed. EFSA J. 9, 2438. https://doi.org/10.2903/j.efsa.2011.2438.

Enzing, C., Ploeg, M., Barbosa, M., Sijtsma, L., 2014. Micro-algal production systems, Microalgae-based products for the food and feed sector: an outlook for Europe. JRC scientific and policy reports; No. JRC 85709. https://doi.org/10.2791/3339.

FDA, 2003. GRAS Notice GRN 127. Spirulina, the Dried Biomass of *Arthrospira Platensis*. Cyanotech Corporation and Earthrise Nutritionals, Inc. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=127, Accessed date: 15 April 2019.

FDA, 2012a. GRAS Notice GRN 384. Algal Oil Derived from *Chlorella Protothecoides* Strain S106 (Cp Algal Oil). Solazyme, Inc. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=384, Accessed date: 15 April 2019.

FDA, 2012b. GRAS Notice GRN 394. Dried Biomass of *Arthrospira Platensis*, Also Known as Spirulina Platensis (Spirulina). RFI, Inc. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=394, Accessed date: 15 April 2019.

FDA, 2012c. GRAS Notice GRN 417. Dried Biomass of *Arthrospira Platensis* Also Known as Spirulina Platensis (Spirulina). E.I.D. Parry (India) Ltd. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=417, Accessed date: 15 April 2019.

FDA, 2013. GRAS Notice GRN 469. *Chlorella Protothecoides* Strain S106 Flour with 40-70% Lipid (Algal Flour). Solazyme Roquette Nutritionals, LLC. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=469, Accessed date: 15 April 2019.

FDA, 2014. GRAS Notice GRN 519. *Chlorella Protothecoides* Strain S106 Flour with 40-75% Protein. Solazyme, Inc. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=519, Accessed date: 15 April 2019.

Goiris, K., Muylaert, K., Fraeye, I., Foubert, I., De Brabanter, J., De Cooman, L., 2012. Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. J. Appl. Phycol. 24, 1477–1486. https://doi.org/10.1007/s10811-012-9804-6.

Hallmann, A., 2007. Algal transgenics and biotechnology. Transgenic Plant J. 1, 81–98.

Health Canada, 2018. Novel food information – lyophilized Tetraselmis chui strain 8/6 as a novel food. 2018. https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/approved-products/novel-food-information-lyophilized-tetraselmis-chui-strain-8-6.html, Accessed date: 24 April 2019.

Himuro, S., Ueno, S., Noguchi, N., Uchikawa, T., Kanno, T., Yasutake, A., 2017. Safety evaluation of *Chlorella sorokiniana* strain CK-22 based on an *in vitro* cytotoxicity assay and a 13-week subchronic toxicity trial in rats. Food Chem. Toxicol. 106, 1–7. https://doi.org/10.1016/j.fct.2017.05.025.

Intergovernmental Institution for the Use of Micro-Algae Spirulina Against Malnutrition (IIMSAM) http://iimsam.org/, Accessed date: 16 April 2019.

Kagan, M.L., Sullivan, D.W., Gad, S.C., Ballou, C.M., 2014. Safety assessment of EPA-rich polar lipid oil produced from the microalgae *Nannochloropsis oculata*. Int. J. Toxicol. 33, 459–474. https://doi.org/10.1177/1091581814553453.

Lafarga, T., Acién-Fernández, F.G., Castellari, M., Villaró, S., Bobo, G., Aguiló-Aguayo, I., 2019. Effect of microalgae incorporation on the physicochemical, nutritional, and sensorial properties of an innovative broccoli soup. Lebensm. Wiss. Technol. 111, 167–174. https://doi.org/10.1016/j.lwt.2019.05.037.

Lavens, P., Sorgeloos, P., 1996. Use of microalgae. In: Manual on the Production and Use of Live Food for Aquaculture, vol. 361. FAO Fisheries Technical Paper, pp. 36–40. https://doi.org/10.1017/CBO9781107415324.004.

Li, H.Y., Yan, X.J., Xu, J.L., Zhou, C.X., 2008. Precise identification of photosynthetic glycerolipids in microalga *Tetraselmis chuii* by UPLC-ESI-Q-TOF-MS. Sci. China Ser. C Life Sci. 51, 1101–1107. https://doi.org/10.1007/s11427-008-0138-1.

Liu, J., Chen, F., 2014. Biology and industrial applications of Chlorella: advances and prospects. Adv. Biochem. Eng. Biotechnol. 153, 1–35. https://doi.org/10.1007/10_2014_286.

Llana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Pichardo, S., Jos, A., Moyano, R., Cameán, A.M., 2017. A subchronic 90-day oral toxicity study of *Origanum vulgare* essential oil in rats. Food Chem. Toxicol. 101, 36–47. https://doi.org/10.1016/j.fct.2017.01.001.

Lonza, 2019. Lonza launches Oceanix™, a CleanLabel ingredient for peak performance and recovery. Available at: http://e3.marco.ch/publish/lonza/551_931/190305_OCEANIX_FINAL.pdf.

Matulka, R.A., Chan, T., Green, R., Carney, J.R., Franklin, S., Licari, P., 2016. 13-week dietary study and *in vitro* and *in vivo* genotoxicity studies of a structuring fat produced through a microalgal fermentation process. Toxicol. Rep. 3, 123–134. https://doi.org/10.1016/j.toxrep.2015.12.006.

Mellado-García, P., Puerto, M., Pichardo, S., Llana-Ruiz-Cabello, M., Moyano, R., Blanco, A., Jos, A., Cameán, A.M., 2016. Toxicological evaluation of an Allium-based commercial product in a 90-day feeding study in Sprague-Dawley rats. Food Chem. Toxicol. 90, 18–29. https://doi.org/10.1016/j.fct.2016.01.019.

Mourente, G., Lubián, L.M., Odriozola, J.M., 1990. Total fatty acid composition as a taxonomic index of some marine microalgae used as food in marine aquaculture. Hydrobiologia 203, 147–154. https://doi.org/10.1007/BF00005683.

Muller-Feuga, A., 2000. The role of microalgae in aquaculture: situation and trends. J. Appl. Phycol. 12, 527–534. https://doi.org/10.1023/A:100810630.

Muller-Feuga, A., Robert, R., Cahu, C., Robin, J., Divanach, P., 2007. Uses of microalgae in aquaculture. In: Live Feeds in Marine Aquaculture, (Chapter 7). https://doi.org/10.1002/9780470995143.ch7.

Natrah, F.M.I., Yusoff, F.M., Shariff, M., Abas, F., Mariana, N.S., 2007. Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value. J. Appl. Phycol. 19, 711–718. https://doi.org/10.1007/s10811-007-9192-5.

OECD (Organisation for Economic Cooperation and Development), 1998. Guideline for testing of chemicals. Repeated dose 90-day oral toxicity study in rodents. Guidelines 408, 1–10.

OECD (Organisation for Economic Cooperation and Development), 2018. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents, OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264070707-en.

OECD (Organisation for Economic Cooperation and Development), 2001. Guideline for testing of chemicals. Acute Oral Toxic. - Acute Toxic Class Method, Guidel. 423, 1–14. https://doi.org/10.1787/20745788.

Regulation (EC) No. 258/97 of the European Parliament and of the Council of 27 January 1997 Concerning Novel Foods and Novel Foods Ingredients. OJ L043, 14.01. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A31997R0258.

Regulation (EU) 2015/2283 of the european parliament and of the Council of 25 november 2015 on novel foods, amending regulation (EU) N. 1169/2011 of the european parliament and of the Council and repealing regulation (EC) No 258/97 of the european parliament and of the Council and commission regulation (EC) No 1852/2001. OJ L327/1, 11.12. https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX%3A32015R2283.

Rodriguez-Garcia, I., Guil-Guerrero, J.L., 2008. Evaluation of the antioxidant activity of three microalgal species for use as dietary supplements and in the preservation of foods. Food Chem. 108, 1023–1026. https://doi.org/10.1016/j.foodchem.2007.11.

059.
Safafar, H., Wagenen, J., Van Møller, P., Jacobsen, C., 2015. Carotenoids, phenolic compounds and tocopherols contribute to the antioxidative properties of some microalgae species grown on industrial wastewater. Mar. Drugs 13, 7339–7356. https://doi.org/10.3390/md13127069.
Schmitt, D., Tran, N., Peach, J., Bauter, M., Marone, P., et al., 2012. Toxicologic evaluation of DHA-rich algal oil: Genotoxicity, acute and subchronic toxicity in rats. Food and Chemical Toxicology 50 (10), 3567–3576. https://doi.org/10.1016/j.fct.2012.07.054.
Simon, R.R., Vo, T.D., Levine, R., 2016. Genotoxicity and subchronic toxicity evaluation of dried *Euglena gracilis* ATCC PTA-123017. Regul. Toxicol. Pharmacol. 80, 71–81. https://doi.org/10.1016/j.yrtph.2016.06.007.
Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., 2006. Commercial applications of microalgae. J. Biosci. Bioeng. 101, 87–96. https://doi.org/10.1263/jbb.101.87.
Symonds, J.M., Beauchamp, N., Takeuchi, T., Yamada, K., Atsuji, K., Asayama, Y., Suzuki, K., Kruger, C.L., 2018. Toxicological assessment of *Euglena gracilis* strain Eu029 shows no adverse effects *in vivo* and *in vitro*. Toxicol. Res. Appl. 2, 1–11. https://doi.org/10.1177/2397847318761672.
Szabo, N.J., Matulka, R.A., Chan, T., 2013. Safety evaluation of whole Algalin protein (WAP) from *Chlorella protothecoides*. Food Chem. Toxicol. 59, 34–45. https://doi.org/10.1016/j.fct.2013.05.035.
Szabo, N.J., Matulka, R.A., Marone, P.A., Bauter, M.R., Chan, T., Franklin, S., Carney, J.R., McQuaid, S.L., Rakitsky, W., Green, R., Licari, P., 2014. Safety evaluation of oleic-rich triglyceride oil produced by a heterotrophic microalgal fermentation process. Food Chem. Toxicol. 65, 301–311. https://doi.org/10.1016/j.fct.2013.12.048.
Takaichi, S., 2011. Carotenoids in algae: distributions, biosyntheses and functions. Mar. Drugs 11, 1101–1118. https://doi.org/10.3390/md9061101.
Turck, D., Bresson, J., Burlingame, B., Dean, T., Fairweather-Tait, S., Heinonen, M., Hirsch-Ernst, K.I., Mangelsdorf, I., McArdle, H., Naska, A., Neuhäuser-Berthold, M., Nowicka, G., Pentieva, K., Sanz, Y., Siani, A., Sjödin, A., Stern, M., Tomé, D., Vinceti, M., Willatts, P., Engel, K., Marchelli, R., Pöting, A., Poulsen, M., Salminen, S., Schlatter, J., Arcella, D., Gelbmann, W., de Sesmaisons-Lecarré, A., Verhagen, H., van Loveren, H., 2016. Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. EFSA J., 14. https://doi.org/10.2903/j.efsa.2016.4594.