ORIGINAL SUBMISSION

673

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Orlando, Florida 32814
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September 23, 2016

Paulette M. Gaynor, Ph.D.
Deputy Division Director
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

RE: Algal Structuring Fat GRAS Notification

Dear Dr. Gaynor:

In accordance with 21 CFR §170, Subpart E – Generally Recognized As Safe (GRAS) Notice, I am submitting, as the agent of the notifier, TerraVia Holdings, Inc. (TerraVia, previously known as Solazyme, Inc.), 225 Gateway Blvd. South San Francisco, CA 94080 a notification of the conclusion of GRAS status for the use of stearic-oleic-stearic algal structuring fat (algal structuring fat) as a partial replacement for conventional dietary fats or oils in the diet at an estimated upper consumption level of 9.10 g/day, typically used in the U.S. to represent a long-term or "lifetime averaged" daily intake estimate.

Best regards,

Ray A. Matulka, Ph.D.
Director of Toxicology

RECEIVED
OCT 6 2016
OFFICE OF
FOOD ADDITIVE SAFETY

Form Approved: OMB No. 0910-0342; Expiration Date: 03/31/2019
(See last page for OMB Statement)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000673	OCT 6, 2016
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

PART I – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. ________ ☐ Supplement to GRN No. ________

2. ☐ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3a. For New Submissions Only: Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: ________

3b. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: ________

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position
See Agent	See Agent

Company *(if applicable)*: TerraVia Holdings, Inc.

Mailing Address *(number and street)*: 225 Gateway Blvd

City	State or Province	Zip Code/Postal Code	Country
South San Francisco	California	94080	United States of America

Telephone Number	Fax Number	E-Mail Address
407.802.1400		

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position
Ray A. Matulka, Ph.D.	Director of Toxicology

Company *(if applicable)*: Burdock Group Consultants

Mailing Address *(number and street)*: 859 Outer Road

City	State or Province	Zip Code/Postal Code	Country
Orlando	Florida	32814	United States of America

Telephone Number	Fax Number	E-Mail Address
407.802.1400	407.802.1405	rmatulka@burdockgroup.com

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

Algal Structuring Fat

2. Submission Format: *(Check appropriate box(es))*

- ☐ Electronic Submission Gateway
- ☒ Paper
- ☐ Electronic files on physical media with paper signature page

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes 1

Total number of pages 74

4. Does this submission incorporate any information in FDA's files by reference? *(Check one)*

☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates by reference information from a previous submission to FDA as indicated below *(Check all that apply)*

- ☒ a) GRAS Notice No. GRN 527
- ☐ b) GRAS Affirmation Petition No. GRP
- ☐ c) Food Additive Petition No. FAP
- ☐ d) Food Master File No. FMF
- ☒ e) Other or Additional *(describe or enter information as above)* GRN 384, 469, and 519

6. Statutory basis for determination of GRAS status *(Check one)*

☒ Scientific Procedures *(21 CFR 170.30(b))* ☐ Experience based on common use in food *(21 CFR 170.30(c))*

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?

- ☒ Yes *(Proceed to Item 8)*
- ☐ No *(Proceed to Part IV)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

- ☐ Yes, see attached Designation of Confidential Information
- ☒ Yes, information is designated at the place where it occurs in the submission
- ☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

- ☐ Yes, a redacted copy of the complete submission
- ☒ Yes, a redacted copy of part(s) of the submission
- ☐ No

PART IV – INTENDED USE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance *(e.g., when a substance would be an ingredient in infant formula, identify infants as a special population).*

Algal structuring fat may be used as a partial replacement for stearic acid-containing dietary fats or oils in a variety of conventional food groups (Margarine and margarine-like spreads, butter-like spreads, vegetable shortenings, nut spreads, milk products, non-dairy products, baked goods, chocolate-based sauces and syrups, baked desserts, cookies and frozen dairy desserts) that contain fats that are solid at room temperature, resulting in an estimated 90th percentile consumption of 9.10 g/day by the general U.S. population.

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? *(Check one)*

☒ Yes ☐ No

PART V – IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used *(CAS, EC)*	Registry No.[2]	Biological Source *(if applicable)*	Substance Category *(FOR FDA USE ONLY)*
1	Algal structuring fat			The microalgae *Prototheca moriformis*	
2					
3					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (1 - 3) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s))*, and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

The algal structuring fat is a pale yellow to wheat yellow-colored, refined, bleached and deodorized fat isolated from an engineered strain of the microalga *P. moriformis*. The neutral-flavored oil is almost exclusively in the form of triglycerides (≥ 95%) with small amounts of diglycerides (< 5%) and trace amounts of monoglycerides (< 0.5%). Small quantities of free fatty acids (≤ 0.1%) and polar compounds (< 4%) are also present. Non-saponifiable matter comprises ≤ 1% of the oil, with moisture being ≤ 0.1%. Stearic acid is the predominant fatty acid (≥ 50 Area % Total Fatty Acid (TFA)) followed by oleic (~30 Area % TFA) and palmitic acids (~2 Area % TFA) and lesser amounts of other fatty acids.

3. Synonyms

Provide as available or relevant:

1	Stearic-oleic-stearic algal structuring fat
2	Algae structuring fat
3	Algal butter

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE
(check list to help ensure your submission is complete – check all that apply)

- [x] Any additional information about identity not covered in Part V of this form
- [x] Method of Manufacture
- [x] Specifications for food-grade material
- [x] Information about dietary exposure
- [x] Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- [x] Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- [x] Comprehensive discussion of the basis for the determination of GRAS status
- [x] Bibliography

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

[x] Yes [ ] No

Did you include this other information in the list of attachments?

[x] Yes [ ] No

PART VII – SIGNATURE

1. The undersigned is informing FDA that TerraVia Holdings, Inc.
(name of notifier)

has concluded that the intended use*(s)* of Algal Structuring Fat
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. [x] See Agent *(name of notifier)* agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.

See Agent *(name of notifier)* agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so.

859 Outer Road, Orlando, FL 32814
(address of notifier or other location)

See Agent *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

[ ] The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No.

(GRAS Affirmation Petition No.)

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
	Ray A. Matulka, Ph.D., Director of Toxicology	09/27/2016

PART VIII – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	GRAS Notification	1-74

OMB Statement: Public reporting burden for this collection of information is estimated to average 150 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, 1350 Piccard Drive, Room 400, Rockville, MD 20850. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

F. Freedom of Information Act Exemption

Select information in Part 6 of this GRAS notice is exempt from disclosure under the Freedom of information Act, 5 U.S.C. 522, as the selected information (that has been blocked from view) is commercial information that is privileged and confidential. Part 6 of the notification includes the redaction of confidential information concerning specific heating temperatures and times during the manufacturing process, the specific names and sequences of genes that were modified or deleted (while still retaining an explanation of the function and source of the genes, and therefore providing sufficient information for the conclusion of GRAS status), growth conditions and media components. All media components have either been previously determined GRAS, are food additives, or were safe and suitable for its intended use, and therefore the redaction of the specific names of these media components would not affect the conclusion of GRAS of this algal structuring fat. Overall, the redaction of this information would not limit the ability of experts in the field of food ingredient toxicology to conclude that algal structuring fat is GRAS under its intended conditions of use.

G. Certification

The undersigned author of this document hereby certifies that, to the best of their knowledge, this document is a complete, representative and balanced representation of all available information, favorable as well as unfavorable, known by the author to be pertinent to the evaluation of the safety and GRAS status of the use of the substance.

Signed,

(b) (6)

Date September 23, 2016

Ray A. Matulka, Ph.D.
Director of Toxicology
Burdock Group
859 Outer Road
Orlando, FL 32814
Telephone: 407-802-1400
Facsimile: 407-802-1405
Email: rmatulka@burdockgroup.com

1. Signed Statements and Certification

In accordance with 21 CFR §170, Subpart E – Generally Recognized As Safe (GRAS) Notice, I, Ray A. Matulka, Ph.D., am submitting a GRAS notification for the use of Algal Structuring Fat as specified in this notification as the agent of the notifier [TerraVia Holdings, Inc. (formerly known as Solazyme, Inc.)].

Notifier:

TerraVia Holdings, Inc.
(formerly known as Solazyme, Inc.)
225 Gateway Blvd
South San Francisco, CA 94080

Agent of the Notifier:

Ray A. Matulka, Ph.D.
Director of Toxicology
Burdock Group
859 Outer Road
Orlando, FL 32814
Telephone: 407-802-1400
Facsimile: 407-802-1405
Email: rmatulka@burdockgroup.com

A. Name of the Notified Substance

For the purposes of this GRAS Notification, the name used to describe the ingredient is:

Algal Structuring Fat

However, the common and usual name for the ingredient is Algae Butter. Algae Butter is appropriate for consumer labeling as it can be a replacement for commonly used substances with similar structure, function, and uses (*i.e.*, fat containing butters such as cocoa butter or shea butter). Synonyms include, but are not limited to: stearic-oleic-stearic algal structuring fat, algae structuring fat, and algal butter.

B. Conditions of Use

Algal structuring fat may be used as a partial replacement for stearic acid-containing dietary fats or oils in a variety of conventional food groups (Table 1) that contain fats that are solid at room temperature,[1] none of which have a standard of identity,[2] resulting in an estimated 90th percentile consumption level of 9.10 g/day by the general U.S. population.

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[1] Margarine and margarine-like spreads, butter-like spreads, vegetable shortenings, nut spreads, milk products, non-dairy products, baked goods, chocolate-based sauces and syrups, baked desserts, cookies and frozen dairy desserts.

[2] All food categories designated by TerraVia have been utilized in the estimated dietary intake calculations as appropriate; however, certain categories designated by TerraVia may contain foods for which a standard of identity exists. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity. TerraVia confirms that the algal structuring fat will be added only to foods for which a standard of identity does not exist or to those foods with a standard of identity that does not specify a particular fat.

Table 1. Food groups selected for algal structuring fat supplementation*

Food Category	Maximum intended use level (ppm)
Margarine and margarine-like spreads (12)	150,000
Butter-like spreads (12)	200,000
Vegetable shortenings (12)	250,000
Nut spreads (32)	20,000
Milk products (31)	20,000
Non-dairy products (10)	250,000
Baked goods (1)	100,000
Chocolate-based sauces and syrups (43)	10,000
Baked desserts (1)	30,000
Cookies (1)	50,000
Frozen dairy desserts (20)	20,000

*The food categories correspond to those listed in 21 CFR §170.3(n). The number in parenthesis following each food category is the paragraph listing in 21 CFR §170.3(n) for that food category.
ppm = parts *per* million.

C. Basis of GRAS Determination

Pursuant to 21 CFR §170.3, a conclusion of GRAS status through scientific procedures, in accordance with 170.30(a) and (b), was found for the use of algal structuring fat as an ingredient in food for its intended conditions of use.

D. Premarket Approval Exemption

TerraVia Holdings, Inc. (TerraVia, previously known as Solazyme, Inc.), 225 Gateway Blvd. South San Francisco, CA 94080 has concluded that stearic-oleic-stearic algal structuring fat (algal structuring fat), derived from a non-toxigenic classically and genetically modified strain of *Prototheca moriformis*, is generally recognized as safe (GRAS) as a food ingredient and therefore, exempt from the requirement of premarket approval of the Federal Food, Drug and Cosmetic Act and from environmental impact, under the conditions of its intended use.

E. Availability of Information

The data and information that serve as a basis for this GRAS determination are available for FDA review and copying at reasonable times at:

Burdock Group
859 Outer Road
Orlando, FL 32814
Telephone: 407-802-1400
Facsimile: 407-802-1405
Email: rmatulka@burdockgroup.com

Alternatively, a copy of the data and information that serve as a basis for this GRAS conclusion may be sent *via* electronic format or on paper to FDA upon request.

H. United States Department of Agriculture, Food Safety Inspection Service Review

Where applicable, as required by §170.270, FDA is authorized to send any trade secrets to the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA) or ask FDA to exclude any trade secrets from the copy of the GRAS notice that will be sent to FSIS.

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2. Detailed Information about the Identity of the Notified Substance

A. Identity of Algal Structuring Fat

The algal structuring fat is a pale yellow to wheat yellow-colored, refined, bleached and deodorized fat isolated from an engineered strain of the microalga *P. moriformis*. Typical physical and chemical properties of the high-oleic algal oil are provided in Table 2. The neutral-flavored oil is almost exclusively in the form of triglycerides (≥ 95%) with small amounts of diglycerides (< 5%) and trace amounts of monoglycerides (< 0.5%). Small quantities of free fatty acids (≤ 0.1%) and polar compounds (< 4%) are also present. Non-saponifiable matter comprises ≤ 1% of the oil, with moisture being ≤ 0.1%. Stearic acid is the predominant fatty acid (≥ 50 Area % Total Fatty Acid (TFA)) followed by oleic (~30 Area % TFA) and palmitic acids (~2 Area % TFA) and lesser amounts of other fatty acids.

Table 2. Typical physical and chemical properties of the algal structuring fat

Characteristic	Value
Synonyms	Algal structured fat; SOS algal fat
Appearance	Pale yellow to wheat yellow solid
Smoke point	201 ± 4ºC
Flash point	227 ± 5ºC
Neutral oil	99 ± 0.3 %
Fatty acid profile	
C18:0	≥ 50 Area %
C18:1	≥ 30 Area %
C18:2	≤ 2 Area %
C18:3 *alpha*	≤ 0.8 Area %
Total saturates	≥ 50 Area %
Free fatty acids	≤ 1.0 %
Moisture content*	≤ 250 ppm
Unsaponifiable matter	≤ 2 %
Insoluble matter	≤ 0.1 %
Triglycerides	≥ 95.0 %
Diglycerides	≤ 5.0 %
Monoglycerides	≤ 0.5 %
Total polar compounds	< 5.0 %

C18:0 = Stearic acid; C18:1 = Oleic acid; C18:2 = Linoleic acid; C18:3 *alpha* = *alpha*-Linolenic acid; SOS = stearic-oleic-stearic; *Karl Fisher moisture, determined by AOCS Ca 2e-84 and stated in parts per million (ppm)

A.1. Composition of Algal Structuring Fat

The fatty acid composition of the algal structuring fat includes approximately 55% stearic acid and 35% oleic acid of the total fatty content, and is similar to other stearic acid-rich plant-derived solid fats utilized in the food supply. See Table 3 for more details on the levels of different fatty acids contained in the algal structuring fat, as compared to other structuring fats.

Table 3. Fatty acid composition (as a percentage of the total fatty acid content) for three lots of the algal structuring fat compared with conventional structured fat.

Fatty Acids (Area %)	Lot # RBD734	Lot # RBD735	Lot # RBD736	Mean ($n = 3$)	Kokum butter [b]	Shea Stearin [c]	Oil, Shea nut [d]	Oil, Cocoa butter [d]
C8:0	ND	ND	ND	ND	ND	ND	0.20	0.00
C10:0 (Capric)	0.05	0.04	0.08	0.06	0.01	0.02	0.20	0.00
C11:0	ND	ND	ND	ND	ND	ND	NR	NR
C12:0 (Lauric)	0.23	0.24	0.35	0.27	0.05	0.17	1.30	0.00
C12:1	ND	ND	ND	ND	ND	ND	NR	NR
C13:0	ND	ND	ND	ND	ND	ND	NR	NR
C14:0 (Myristic)	0.52	0.54	0.61	0.56	0.04	0.09	0.10	0.10
C14:1, *cis*-9 (Myristoleic)	ND	ND	ND	ND	ND	ND	NR	NR
C15:0 (Pentadecanoic)	0.01	0.02	0.01	0.01	0.01	ND	NR	NR
C16:0 (Palmitic)	4.15	4.22	4.35	4.24	2.05	3.16	4.40	25.40
C16:1, *cis*-7	0.07	0.07	0.07	0.07	0.02	ND	0.10	0.20
C16:1, *cis*-9 (Palmitoleic)	0.06	0.07	0.07	0.07	0.01	0.02	NR	NR
C17:0 (Heptadecanoic)	0.09	0.09	0.09	0.09	0.11	0.10	NR	NR
C17:1, *cis*-9	0.05	0.01	0.05	0.04	ND	ND	NR	NR
C18:0 (Stearic)	55.53	55.12	54.59	55.08	57.47	57.73	38.80	33.20
C18:1, *cis*-9 (Oleic)	34.83	34.98	35.02	34.94	38.18	33.05	43.50	32.60
C18:2, *cis*-9,12 (Linoleic)	1.26	1.29	1.31	1.29	0.83	2.85	4.90	2.80
C18:3 *alpha*	0.24	0.28	0.28	0.27	0.08	0.05	0.30	0.10
C20:0 (Arachidic)	1.39	1.36	1.34	1.36	0.47	1.74	NR	NR
C20:1	0.10	0.10	0.10	0.10	0.18	0.11	0.00	0.00
C20:2	0.02	0.03	0.02	0.02	ND	ND	NR	NR
C20:3n3	0.01	0.01	ND	0.01 [a]	ND	ND	NR	NR
C21:0	ND	ND	ND	ND	ND	ND	NR	NR
C22:0 (Behenic)	0.14	0.14	0.14	0.14	0.03	0.16	NR	NR
C22:1, *cis*-13 (Erucic)	ND	ND	ND	ND	ND	ND	0.00	0.00
C22:2n6	0.08	0.10	0.09	0.09	ND	0.04	NR	NR
C23:0 (Tricosanoic)	ND	ND	ND	ND	ND	ND	NR	NR
C24:0 (Lignoceric)	0.07	0.08	0.06	0.07	ND	0.11	NR	NR
Total Fatty Acid	98.90	98.77	98.63	98.77	99.54	99.40	93.80	94.40
TAG Profiles								
Saturate-Saturate-Saturate (Sat-Sat-Sat)	4.79	4.52	4.35	4.55	0.82	2.40	NA	NA
Saturate-Unsaturate-Saturate (Sat-Unsat-Sat)	79.38	80.42	79.93	79.91	83.42	88.80	NA	NA
Saturate-Oleic-Saturate (Sat-O-Sat)	76.44	77.44	76.75	76.88	81.66	82.70	NA	NA
Melting Point (°C)	46.8	46.9	46.1	46.6	37.0 – 40.0			

[a] $n = 2$.

[b] Obtained from Essential Wholesale & Labs and refined, bleached, and deodorized.

[c] Obtained from Archer Daniel Midlands (ADM) Corporation.

[d] USDA National Nutrient Database for Standard Reference, Release 27 (<http://ndb.nal.usda.gov/ndb/search/list>; site last accessed June 26, 2015).

n = number; NA = Not analyzed; ND = Analyzed, but not detected; NR = Not reported; TAG = Triacylglyceride.

A.2. Source Organism Taxonomic Analysis

Algal structuring fat is isolated from a strain of *Prototheca moriformis* that had been modified *via* classical mutagenesis and targeted genetic modification (designated by TerraVia as strain S7737). The genus *Prototheca*, which is composed of achlorophyllous[3] eukaryotic microalgae, belongs to the Trebouxiophyceae class in phylum Chlorophyta. The related *Chlorella* genus is also a member of the same class in the same phylum. Although *Prototheca* are occasionally referred to as 'colorless *Chlorella*' due their lack of chloroplasts and photosynthetic pigments and close relationship with *C. protothecoides* (aka, *Auxenochlorella protothecoides*), species within the *Prototheca* genus have several additional differentiating traits when compared with *Chlorella* spp.: (1) *Prototheca* spp. are acidophilic, (2) *Prototheca* spp. are thiamine auxotrophs, and (3) *Prototheca* spp. cannot utilize nitrate as a sole nitrogen source, but can use a variety of hydrocarbons as sole carbon sources (Running *et al.*, 2003b). Of all the heterogeneous species that comprise the *Chlorella* genus, only *C. protothecoides* shares most of these distinguishing characteristics and is recognized to be particularly closely related to *Prototheca* spp. (Conte and Pore, 1973; Running *et al.*, 2003b; Ewing *et al.*, 2014).

Prototheca are spherical or ovoid unicellular heterotrophic eukaryotes (3 – 30 µm in diameter) having double-layered cell walls that, similar to *C. protothecoides*, contain sporopollenin.[4] These achlorophyllous microalgae lack chloroplasts and buds (Lass-Florl and Mayr, 2007; Hillesheim and Bahrami, 2011; Mayorga *et al.*, 2012). Reproduction is by asexual endosporulation in which two to 16 endospores (2 – 10 µm in diameter), symmetrically or randomly arranged in the sporangia, develop until they emerge *via* rupture of the parent sporangial cell wall (DiPersio, 2001; Lass-Florl and Mayr, 2007; Hillesheim and Bahrami, 2011; Mayorga *et al.*, 2012). In the presence of adequate nutrients, reproductive cycles can occur every 5 – 6 hours with the resulting release of sporangiospores (Lass-Florl and Mayr, 2007). *P. moriformis* (including strain S7737) and its sporangiospores are highly sensitive to inactivation by heat or chemical methods.

Prototheca spp. are ubiquitous in the environment. Although first isolated between 1892 – 1894 from the slime fluxes (*i.e.*, sap released from tree wounds) of lime, horse chestnut and elm trees in Germany by W. Kruger (Krüger, 1894; Pore *et al.*, 1983; 1985), *Prototheca* spp. have since been isolated from a wide variety of sources,[5] including environmental sources such as soil, decaying plant matter, sewage, and water, as well as from human and animal sources (*e.g.*, feces, milk, sputum, and/or lesions) (Mayorga *et al.*, 2012).

The number of species assigned to the *Prototheca* genus has varied over the years as the understanding of the phylogenetic relationships between species has increased. A majority of the scientific literature currently recognizes five species in the genus: *P. wickerhamii*, *P. zopfii*, *P. stagnora*, *P. ulmea*, and *P. blaschkeae* (Roesler *et al.*, 2006; Marques *et al.*, 2008; Mayorga *et al.*, 2012). Arholdt *et al.* (2012) recognizes *P. cutis* as a sixth. *P. moriformis* has at times been considered a species of *Prototheca* (Pore, 1985; Roesler *et al.*, 2003; Ueno *et al.*, 2005; Jagielski and Lagneau, 2007), but the assignment has not been generally agreed upon as valid (Pore, 1985;

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[3] Achlorophyllous = without photosynthetic pigments.

[4] Rare but natural oxidative carotenoid polymers that occur in a few microorganisms and plants.

[5] Of 100 randomly selected strains of *Prototheca* held in the private culture collection belonging to Professor R. Scott Pore at the West Virginia University Medical School, Charleston WV), seven had been isolated from humans, 35 from animals, and 58 from the environment (Shahan and Pore, 1991; Running *et al.*, 2003a).

Roesler *et al.*, 2003; Ueno *et al.*, 2003; 2005; Lass-Florl and Mayr, 2007; Marques *et al.*, 2008; Mayorga *et al.*, 2012). Recent molecular phylogenetic data shows a strong affinity between *P. wickerhamii* and *P. moriformis* groups (Ewing *et al.*, 2014).

Recent taxonomic analysis clearly differentiates the *Prototheca* species into five distinct clades, which are composed of: (A) *A. protothecoides*; (B) A single *Prototheca wickerhamii* isolate; (C) A mixture of *P. wickerhamii* and *P. moriformis* isolates (including UTEX 1435, the wild type strain pertinent to this dossier); (D) *P. ulmea* and *P. stagnora*; and (E) primarily *P. zopfii* isolates with *P. moriformis*, as well as *P. kruegani* and *P. blaschkeae* (Ewing *et al.*, 2014). The authors, who examined a larger number of *Prototheca* isolates than had been included in most of the earlier studies, concluded that strains of *P. moriformis, P. wickerhamii*, and *P. stagnora* are part of paraphyletic groups that still require additional resolution.

Of the currently recognized species of *Prototheca*, *P. zopfii, P. wickerhamii, and P. blaschkeae*, each contain strains that have been documented as opportunistic pathogens causing rare infections (*i.e.*, protothecosis) in humans and domestic and wild animals. *P. wickerhamii* is most often associated with the rare infections reported in humans. In a typical year, two to five cases of infection are reported globally; from 1964 – 2012, a total of 160 cases of human protothecosis have been reported (Todd *et al.*, 2012). Infection in humans is often associated with traumatic introduction into the skin and underlying tissues, and systemic infection is usually associated with severe immunosuppression or underlying disease (Lass-Florl and Mayr, 2007; Mayorga *et al.*, 2012). *P. zopfii* genotype 2 is most often associated with infection in animals, primarily bovine mastitis in cows and occasionally infections in dogs, but is rarely associated with disease in humans. Nearly all *P. blaschkeae* strains have been isolated from swine, although the first isolate of the species originated from a case of human onychomycosis[6] and the strains are now also associated with bovine mastitis (Roesler *et al.*, 2006; Marques *et al.*, 2008). Bovine protothecal mastitis and indeed, mastitis caused by environmental microorganisms in general, is believed to be primarily a disease of poor hygiene practices and dairy management rather than due to presence or absence of the microorganism, as *Prototheca* spp. have been identified as present in the environments of dairy cattle with and without protothecal mastitis, in the environments of dairy farms with no prior history of mastitis caused by this agent (Anderson and Walker, 1988; Pore and Shahan, 1988; Enders and Weber, 1993; Corbellini *et al.*, 2001; Janosi *et al.*, 2001).

A.3. Wild-type strain *Prototheca moriformis* Krüger

TerraVia obtained wild-type strain *P. moriformis* Krüger UTEX 1435[7] from the University of Texas, Culture Collection of Algae located in Austin, Texas, where it had been deposited by W.B. Cooke in 1966. After undergoing clonal purification to ensure the culture was axenic (free of any contaminating organisms), UTEX 1435 was assigned the TerraVia internal strain number 'S376'. Other deposits of this strain[8] include the ATCC 16525[9] accession held at the American

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[6] 'Fungal' infection of the nail and/or nail bed.

[7] <http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=3871>; site last accessed January 9, 2014.

[8] Although the CDC B-1444 accession deposited into the Center for Disease Control (CDC, Atlanta, Georgia) and the NRRL Y-6865 accession held by the Agriculture Research Service (ARS) Culture Collection (Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois) had been assigned to *P. moriformis* ATCC 16525 as identical strains (Sudman and Kaplan, 1973), they were later determined to be strains of *P. wickerhamii* (Pore, 1985).

[9] <http://www.atcc.org/Products/All/16525.aspx>; site last accessed January 9, 2014.

Type Culture Collection (ATCC, Manassas, Virginia); Hopkins IV 7.3.2.1[17] assigned by C.B. van Niel at the Hopkins Marine Station, Stanford University (Pacific Grove, California) (Sudman and Kaplan, 1973); and the SAG 263-7[10] accession deposited by M.B. Allen held in the Sammlung von Algenkulturen Göttingen (SAG) (Albrecht-von-Haller-Institute for Plant Science, University of Göttingen, Göttingen, Germany). It is generally understood that the original stock of UTEX 1435 was isolated prior to 1894 by W. Krüger from the sap of a lime tree near Jena, Germany and a horse chestnut tree in the botanical gardens at Halle an der Saale in Germany, with the strain most likely originally named *Prototheca zopfii* Krüger[11] (Krüger, 1894) and designated Pr-9 (*Prototheca* 9th strain) when deposited in the collection at the Microbiology Department, Delft Technical School, Delft, The Netherlands. The strain was later provided to C.B. van Niel and deposited in the collection at Hopkins Marine Station (Stanford University, Pacific Grove, CA) where the accession was designated Hopkins IV 7.3.2.1, before being given by van Niel to W.B. Cooke who deposited the strain in UTEX (where the accession was designated *P. moriformis* Krüger UTEX 1435) and in the ATCC (where the accession was designated *P. moriformis* Krüger ATCC 16252). The accession deposited into the SAG by M.B. Allen (designated *P. zopfii* Krüger SAG 263-7) had also been transferred by C.B. van Niel from the Hopkins collection. Recent work however, by Ewing *et al.* (2014), indicates that the SAG accession SAG 263-7 is distinct from UTEX 1435.

While the ATCC preserves *P. moriformis* strain ATCC 16525 under Biosafety Level (BSL) 1 conditions,[10] indicating that the microorganism is not recognized to cause disease in immunocompetent adult humans, the SAG preserves identical accession SAG 263-7 under BSL 2 conditions,[11] which indicates that the microorganism is rarely associated with human disease and of low concern to laboratory staff.

A.4. Modified *Prototheca moriformis*

Mutagenesis of the wild type *P. moriformis* Krüger strain (designated as TerraVia strain S376) was performed to improve yield and productivity of oils the microorganism already produced, as well as to alter oil composition. The steps involved in generating the classically and genetically modified strain of *P. moriformis* used for manufacture of algal structuring fat from the wild-type *P. moriformis* Krüger strain are described in the enclosed GRAS dossier for algal structuring fat and described in more detail in the text that follows.

P. moriformis S376 is a unicellular alga of microscopic size (10 – 12 μm). The strain has a significantly reduced plastid genome compared with *C. protothecoides*, lacks chlorophyll, and is an obligate heterotroph that can metabolize a variety of C6 sugars (*e.g.*, glucose, fructose, galactose, as well as glycerol, but not sucrose) in support of growth. Culture temperatures for growth range from 21.5 – 35.5°C and the strain tolerates pH ranges of 4 – 7. To improve the lipid content *per* cell, overall lipid production (g/l/day) and the yield of oil on carbon substrate, S376 was subjected to classical mutagenesis. The strains resulting from this classical improvement regimen were screened for increased TAG (*i.e.*, triglyceride) accumulation, productivity and yield of oil on carbon substrate. A particularly promising classically modified strain producing increased levels of oleic acid received the TerraVia internal designation 'S5100'. *P. moriformis* strain S5100

[10] <http://sagdb.uni-goettingen.de/detailedList.php?str_number=263-7>; site last accessed January 9, 2014.
[11] During this same time period, a second distinct strain of *Prototheca* was isolated from the slime flux of an elm tree at Halle an der Saale, Germany, and named *Prototheca moriformis* Krüger (Krüger, 1894).

is unable to utilize sucrose as a substrate, consistent with the wild-type *P. moriformis* strain S376 and its other derivatives.

The strain producing the algal structuring fat (S7737) is the result of three separate transformation events, as detailed in the GRAS dossier (TerraVia, 2016). To summarize, the first transformation event was carried out in strain S5100 to generate strain S5780. Strain S5780 was developed by the targeted disruption of an endogenous gene involved in C18:0 fatty acid biosynthesis with integrative vector pSZ2624. As part of the gene disruption cassette, two additional genes were introduced:

(1) an endogenous synthase gene, and

(2) a synthase gene (*ThiC*) from the genus *Arabidopsis*, which serves as a selectable marker and allows the strain to grow in the absence of thiamine.

Intermediary strain S6573 was developed from strain S5780 by transformation with integrative vector pSZ3204 which targets integration via a neutral locus. As part of the integrative cassette, two additional genes were introduced:

(1) a gene encoding for sucrose invertase (*SUC2*) protein from the genus *Saccharomyces* which serves as a second selectable marker and allows the strain to grow on sucrose as a carbon source, and

(2) a gene encoding for a plant thioesterase from the genus *Garcinia.*

Finally, strain S7737 was further developed from strain S6573 by transformation with integrative vector pSZ4164 which targets integration at an endogenous thioesterase involved in fatty acid biosynthesis. As part of the integrative cassette, three additional genes were introduced:

(1) a gene encoding the melibiase (*MEL1*) enzyme which serves as a third selectable marker and allows the strain to grow on melibiose as a carbon source,

(2) a plant transferase gene from the genus *Brassica*, and

(3) a hairpin RNAi cassette targeting an endogenous gene to decrease the triglyceride species of the Stearate-Linoleic-Stearate (SLS) type.

Strain S7737 was selected from among several similarly transformed strains based upon a high stearic acid (> 55%) and low linoleic acid (< 2%) content and superior triglyceride (> 75% Sat-Unsat-Sat; > 75% Sat-O-Sat and < 6% Sat-Sat-Sat) profiles of the resulting finished fat.

B. Method of Manufacture

Algal structuring fat is manufactured by fermenting and harvesting cultures of the engineered *P. moriformis* strain, with the general process depicted in Figure 1. Grown under controlled fermentation conditions, the biomass is prepared by fed-batch axenic fermentation under sterile conditions at a facility compliant with current Good Manufacturing Practice (cGMP). As *per* cGMP, cell banks are prepared to verify the identity and axenic nature of the cultures used in fermentation. Throughout the fermentation process, pH, temperature, agitation, and aeration rates are controlled, as are additions of glucose or sucrose and nutrients, as described in the enclosed GRAS dossier. Following completion of fermentation, the broth is inactivated by heat and concurrently concentrated in an evaporator (≥ 10 hours at 60 °C) and dried (140 – 170 °C, 6 – 15 seconds), after which the dried biomass is mechanically extracted to release the crude algal oil.

The above inactivation step kills all cells, vegetative and endospores. Inactivation occurs at 60 °C with a minimum residence time of 120 seconds. The crude oil is then refined using standard edible oil refining steps, including degumming, bleaching, and deodorization (AOCS, 2011).[12] To ensure stability a food-grade antioxidant may be added prior to packaging, although at present the use of such an agent is not part of the standard process. Except for one substance (*i.e.*, soybean hulls),[13] all ingredients used during manufacture are safe and suitable for human use, as described in the GRAS dossier. Soybean hulls are a fibrous material used as an extraction aid to assist in release of the crude oil from the algal cells.

To support safe use as a press aid, the soybean hulls are assayed for pesticides and potential toxins. Milled soy bean hulls and the final refined, bleached, and deodorized high-oleic algal oil are assayed for pesticides (*i.e.*, polychlorinated biphenyls (PCBs), hexachlorobenzene, methoxychlor, mirex, aldrin, endrin, lindane, chlordane, heptachlor, heptachlor epoxide, benzene hexachloride (BHC), dieldrin, total DDT, parathion, methyl parathion, ethion, methyl trithion, malathion, ronnel, diazinon, disulfeton, and phorate) and mycotoxins (*i.e.*, < 5 ppb[14] aflatoxins, < 25 ppb zearalenone, < 1000 ppb fumonisin, < 25 ppb T-2 toxins, < 2 ppb ochratoxins, and < 500 ppb vomitoxins). No pesticides have been detected and no mycotoxins have been detected above threshold limits.

The final stages of oil manufacture include the refining steps (*i.e.*, degumming, bleaching and deodorization) which efficiently convert the crude oil into the high purity final product. The final product, algal structuring fat, is transferred into 44 gallon drums and stored at room temperature (22 – 27°C) for most distributors, and loaded into totes, bulk trucks and rail cars for large-scale purchase and use.

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[12] Samples of the refined oil product were (1) microscopically examined under 400x magnification; (2) plated in media and incubated alongside negative (media only) and positive controls (1000 CFU of test strain in media) to verify the absence of viable cells; and (3) inoculated in liquid media and incubated alongside negative and positive controls to verify the absence of viable cells. Counts of test plates and negative control plates were negative; counts of positive plates ranged from $10^7 - 10^8$ CFU.

[13] Soybean hulls are approved for use in animal feed (AAFCO, 2015).

[14] ppb = parts *per* billion.

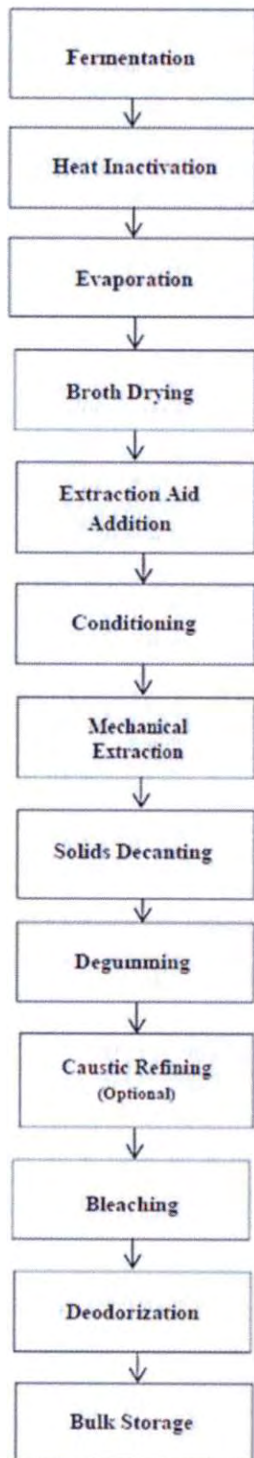

Figure 1. General production process for algal structuring fat.

Regarding labeling concerns related to the eight major food allergens, an extraction aid used in the production of the algal structuring fat is soybean hulls. The soybean hulls used during extraction are not a cause for allergenic concern due to the refining process of the finished algal product. Two closely related high oleic algal oils (also modified from wild type strain *P. moriformis* S376) were manufactured in the same manner as the algal structuring fat of this GRAS dossier. When submitted to the Food Allergy Research and Resource Program at the University of Nebraska (Lincoln, Nebraska) for assay, residual soy allergens were determined in the two crude clarified oils (20 and 25 ppm), but not in the refined, bleached and deodorized (finished) oils (< 2.5 ppm, limit of quantitation) (University of Nebraska, 2013). *Per* the US FDA's Food Allergen Labeling and Consumer Protection Act (FALCPA), a highly refined oil derived from one of the eight foods or food groups is not considered a major food allergen.[15]

C. Specifications

Algal structuring fat for use as a food ingredient is manufactured to meet specifications described in Table 4.

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[15]<http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Allergens/ucm106890.htm#q15>; site last accessed February 19, 2016.

Table 4. Specifications for the algal structuring fat.

Parameter	Method	Specification	Batch Analysis Results (n=3)	
			Range	Average
Appearance	Visual inspection	Pale yellow to wheat yellow solid	Conforms	Conforms
Odor	Olfactory inspection	Slight	Conforms	Conforms
Fatty acid profile	C-M-00036-000 Rev 0[a]			
Palmitic acid (C16:0)	C-M-00036-000 Rev 0[a]	≥ 2 Area %	4.15 – 4.35 Area %	4.24 Area %
Stearic acid (C18:0)	C-M-00036-000 Rev 0[a]	≥ 50 Area %	54.6 – 55.5 Area %	55.1 Area %
Palmitic + Stearic (C16:0 + C18:0)	C-M-00036-000 Rev 0[a]	≥ 55 Area %	58.9 – 59.7 Area %	59.3 Area %
Oleic acid (C18:1)	C-M-00036-000 Rev 0[a]	≥ 30 Area %	34.8 – 35.0 Area %	34.9 Area %
Linoleic acid (C18:2)	C-M-00036-000 Rev 0[a]	≤ 2 Area %	1.26 – 1.31 Area %	1.29 Area %
Alpha-Linolenic acid (C18:3 *alpha*)	C-M-00036-000 Rev 0[a]	≤ 0.8 Area %	0.24 – 0.28 Area %	0.27 Area %
Total Saturated Fat	C-M-00036-000 Rev 0[a]	≥ 50 Area %	61.4 – 62.0 Area %	61.7 Area %
Free Fatty Acids	AOCS Ca 5a-40	≤ 1%	0.38 – 0.58%	0.48%
Unsaponifiable Matter	AOCS Ca 6a-40	≤ 2%	0.63 – 1.07%	0.84%
Peroxide Value	AOCS Cd 8-53	≤ 2 meq/kg	< BLD[b]	< BLD[b]
p-Anisidine Value	ISO 6885	≤ 2%	0.3 – 0.38%	0.35%
Elements by ICP				
Lead	AOCS Ca 17-01	< 0.2 ppm	< 0.20 ppm	< 0.20 ppm
Arsenic	AOCS Ca 17-01	< 0.2 ppm	< 0.20 ppm	< 0.20 ppm
Mercury	AOCS Ca 17-01	< 0.2 ppm	< 0.20 ppm	< 0.20 ppm
Cadmium	AOCS Ca 17-01	< 0.1 ppm	< 0.03 ppm	< 0.03 ppm
Phosphorus	AOCS Ca 20-99	≤ 0.5 ppm	< 0.20 ppm	< 0.20 ppm
Sulfur	AOCS Ca 17-01	≤ 1 ppm	< 0.5 ppm	< 0.5 ppm

[a] C-M-00036-000 Rev 0, Total Deliverable Fatty Acid Methyl Ester Content and Profiles of Algal Oil Samples by Direct Transesterification (internal method).

[b] BLD = Values below the limit of detection = 0.1 meq/kg.

AOCS = American Oil Chemists' Society; ISO = International Standards Organization; meq = milliequivalents; ppm = parts *per* million.

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3. Dietary Exposure

The intake profile (amount and frequency) by individuals in USDA's What We Eat in America (WWEIA) Continuing Survey of Food Intakes by Individuals 2011-2012 (Dwyer *et al.*, 2003)[16] was used to calculate the Estimated Daily Intake (EDI) of algal structuring fat for individuals consuming the food groups selected for the addition of the algal structuring fat *per* this GRAS evaluation (*i.e.*, "eaters only"). The food categories to which algal structuring fat will be added includes bakery, cookie and chocolate-containing food products, and butter/margarine-like products (Table 5). Only certain foods within these categories have been selected; the individual foods selected for addition of the algal structuring fat are provided in GRAS dossier (TerraVia, 2016).

The means and 90th percentile EDIs were calculated only for algal structuring fat intake following addition of algal structuring fat to the selected food groups. The means and 90th percentile EDIs were not calculated for current algal structuring fat intake from natural sources as no information regarding current intakes of algal structuring fat from natural sources was discovered during a comprehensive search of the published literature. Addition of algal structuring fat to the selected foods at the levels specified in the GRAS dossier (TerraVia, 2016) would provide a mean and 90th percentile algal structuring fat consumption of 3.82 and 9.10 g/day, respectively (Table 6).

All food categories designated by TerraVia have been utilized in the calculations as appropriate; however, certain categories designated by TerraVia may include foods for which a standard of identity exists. We note that an ingredient that is lawfully added to food products may be used in a "standardized" food only if it is permitted by the applicable standard of identity. TerraVia confirms that algal structuring fat will be added only to foods for which a standard of identity does not exist.

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[16] USDA (2014) What We Eat In America, NHANES 2011-2012, Dietary intake data fact sheet; Documentation and Data Files. USDA, Agriculture Research Service; http://www.ars.usda.gov/Services/docs.htm?docid=18354; site last accessed December 9, 2015.

Table 5. Food groups selected for algal structuring fat supplementation*

Food Category	Maximum intended use level (ppm)
Margarine and margarine-like spreads (12)	150,000
Butter-like spreads (12)	200,000
Vegetable shortenings (12)	250,000
Nut spreads (32)	20,000
Milk products (31)	20,000
Non-dairy products (10)	250,000
Baked goods (1)	100,000
Chocolate-based sauces and syrups (43)	10,000
Baked desserts (1)	30,000
Cookies (1)	50,000
Frozen dairy desserts (20)	20,000

*The food categories correspond to those listed in 21 CFR §170.3(n). The number in parenthesis following each food category is the paragraph listing in 21 CFR §170.3(n) for that food category.
ppm = parts *per* million.

Table 6. Predicted intake of the algal structuring fat following supplementation of selected foods at the indicated levels (TerraVia, 2016) for individuals consuming selected foods

	***Per* User (g/day)**	
Algal structuring fat intake from:	**Mean**	**90th Percentile**
Possible maximum consumption with algal structuring fat as an added ingredient to food	3.82	9.10

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4. Current, Proposed, and Self-limiting Levels of Use

The algal structuring fat isolated from a modified strain of *P. moriformis* has not been approved for use in food by FDA[17], FEMA[18], or USDA.[19] Although *Prototheca* spp. in a dairy herd environment holds an entry in the FDA Poisonous Plant Database,[20] the entry is generic and has not been updated since 1988. A high oleic algal oil ingredient produced with a related *P. moriformis* strain that underwent classical mutagenesis and targeted genetic modification was determined GRAS, notified to FDA of the GRAS status, and received a "no objection" letter from FDA (GRN 000527).[21]

Algal oil from the related microalga *C. protothecoides* S106 holds GRAS status (GRN 000384)[22] as a food oil to be used in a variety of foods excluding meat and poultry products. In addition, *C. protothecoides* S106 flour with 40 – 70% lipid, called AlgaVia™ Whole Algal Flour (WAF), also holds GRAS status (GRN 000469)[23] as a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening in baked goods, beverages, dairy and egg products, sauces, gravies, margarines, salad dressings, and soups. From the same *C. protothecoides* S106 strain, a high (40 – 75%) protein powder called AlgaVia™ Whole Algal Protein (WAP) has been determined GRAS (GRN 000519)[24] for use as a source of dietary protein in a variety of food products.

Algal structuring fat is a pale yellow to wheat yellow-colored,[25] refined, bleached, and deodorized algal fat consisting of triacylglycerols containing primarily stearic and oleic fatty acids, isolated from a modified strain of *P. moriformis* that will be used to replace a portion of the dietary fats or oils in baked goods, confectionaries, and chocolate-containing products. The levels of intended use range from 10,000 ppm[26] in a variety of foods including cookies and breads to 250,000 ppm in vegetable shortening and powdered cream substitute.

The use of algal structuring fat is self-limiting as excessive addition to a food would cause the food product to be oily to the point on being unpalatable.

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[17] FDA = United States Food and Drug Administration.

[18] FEMA = Flavor Extract Manufactures Associations.

[19] USDA = United States Department of Agriculture.

[20] <http://www.accessdata.fda.gov/scripts/Plantox/Detail.CFM?ID=945>; site last accessed December 22, 2015.

[21] Agency Response Letter GRAS Notice No. GRN 000527; <http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm469231.htm>; site last accessed December 22, 2015.

[22] <http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=384>; site last accessed December 22, 2015.

[23] <http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=469>; site last accessed December 22, 2015.

[24] <http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm449793.htm>; site last accessed November 23, 2015.

[25] The algal structuring fat is not added to food with the intention of acting as a color. Although the algal structuring fat is a pale yellow to wheat yellow in bulk amounts, in the small amounts added to food the color fades to near colorlessness. Under the conditions of maximum usage in foods (up to 250,000 ppm or 25% in shortening, vegetable, and cream substitute, powdered), the color of the food to which the algal structuring fat is added is not altered. The algal structuring fat is, therefore, exempt from the definition of a color additive [FFDCA §201(t) and 21 CFR §70.3(f)] because the ingredient is not effective as a coloring agent.

[26] ppm = parts *per* million.

5. Experience Based on Common Use in Food Before 1958

Algal structuring fat has not been used in food prior to January 1, 1958.

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6. Basis for the Conclusion of GRAS Status

The conclusion that algal structuring fat is GRAS is on the basis of scientific procedures, as described in the Dossier in Support of the Generally Recognized as Safe (GRAS) Status of Stearic-Oleic-Stearic Triglyceride Fat from a Modified Strain of *Prototheca moriformis* (Algal Structuring Fat) as a Food Ingredient, dated April 11, 2016 (TerraVia, 2016).

This part of the notification (i.e., #6 Basis for the Conclusion of GRAS Status) contains information concerning specific heating temperatures and times used during the manufacturing process, the specific names and sequences of genes that were modified or deleted (while still retaining an explanation of the function and source of the genes, and therefore providing sufficient information for the conclusion of GRAS status), growth conditions and media components; because this information is confidential and proprietary and, would otherwise provide competitors with an unfair business advantage if this information was disclosed, the information has been redacted. All media components have either been previously determined GRAS, are food additives, or were safe and suitable for its intended use, and therefore the redaction of the specific names of these media components would not affect the conclusion of GRAS of this algal structuring fat. The following subsections describe the information utilized to conclude that algal structuring fat is GRAS under the intended conditions of use, as concluded by a panel of experts (TerraVia, 2016).

6.1 GRAS Introduction

Cocoa butter has unique melting properties that are difficult to replicate with other natural fats. Cocoa butter's ability to melt at human body temperature, but stay in solid form at room temperature, is derived from its high concentration of "structuring fats". Structuring fats are symmetrical monounsaturated triglycerides in which oleate (C18:1; O) occupies the sn-2 position and palmitic (C16:0; P) and/or stearic (C:18:0; S) acids are in the sn-1 and sn-3 positions, in greater than 70% of the triacylglycerol (TAG) species present (Lipp *et al.*, 2001; Shukla, 2005; De Oliveira *et al.*, 2015). Vegetable fats with such a composition in fat content stay solid at room temperature and raise the melting point to body temperature in chocolate-based formulations where cocoa butter is typically used, while slightly decreasing the tempering time.[27] These fats are termed "cocoa butter equivalents" and include illipe butter,[28] shea butter, kokum butter and sal fat,[29] which contain varying quantities of the 2-oleyl glycerides of palmitic and stearic acid (*e.g.*, POP, POS, SOS), which is a typical property of cocoa butter and make up greater than 70% of the triglyceride profile of cocoa butter (Lipp and Anklam, 1998; Buchgraber *et al.*, 2000; Beppu *et al.*, 2013). Vegetable fat from the seeds of the kokum (*Garcinia indica*) tree is an additional source, but commercial quantities of these fats are limited (Maheshwari and Yella Reddy, 2005). Fractionation and hydrogenation processes, although relatively costly, have been developed to increase levels of structuring fats in vegetable and lauric acid-containing (*e.g.*, palm kernel) oils to impart melt profiles similar to cocoa butter, but the type of TAG species in the starting oil limits food applications (Dijkstra, 2007). Hydrogenation processes used to make cocoa butter-like fats (*e.g.*,

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[27] Tempering of chocolate is the process that re-establishes the cocoa butter crystals that are in solid chocolate; http://www.chocoley.com/resources/about-tempering-chocolate; site last visited February 2, 2016.

[28] Illipe butter is the fat from the nuts of *Shorea stenoptera* (Lipp et al., 2001).

[29] Sal fat is the fat obtained from the seed kernels of *Shorea robusta*, *Shorea stenoptera* and *Madhuca longifolia* trees (Lipp and Anklam, 1998).

converting polyunsaturated fatty acids to monounsaturated and saturated fatty acids) may also generate *trans* fats, as some of the fatty acids are converted to the "*trans*" form. 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 (Klonoff, 2007). *Trans* fats have been found to increase low-density lipoprotein (LDL) levels, increasing the risk of cardiovascular disease (Iqbal, 2014; Ooi *et al.*, 2015). In addition, the United States Food and Drug Administration (FDA) rescinded the Generally Recognized As Safe (GRAS) status of industrially-produced partially hydrogenated oils (PHOs) (FDA, 2015a), limiting any new uses to those petitioned as a food additive requiring premarket approval.

New sources of oils that can either replace or complement currently available oil sources include the use of microalgae that have been found to produce high levels of potentially useful oils (Srirangan *et al.*, 2015). Advances in production and processing have made the manufacture of new microalgal-derived oils and ingredients through heterotrophic fermentation[30] more cost-effective. The ability to genetically engineer certain species of microalgae, as well as tightly control the growth conditions, has resulted in the production of oils with well-defined fatty-acid constituents possessing desirable properties for use in the food industry. *Prototheca moriformis* is an achlorophyllous (*i.e.*, non-chlorophyll producing) microalgae found ubiquitously in the environment and is related to *Chlorella protothecoides* (aka *Auxenochlorella protothecoides*) (Pore *et al.*, 1983). A strain of *P. moriformis* was genetically engineered as a stable microorganism that produces significant amounts of a new structuring fat. Before introducing a new food ingredient to the market for human consumption, a demonstration of the safety of that ingredient must be completed.

The information in this section is a summary of the scientific evidence that supports the GRAS status of a stearic-oleic-stearic algal structuring fat (algal structuring fat) from a modified strain of *P. moriformis* as a human food ingredient.

6.1.1. Description of the Ingredient

Algal structuring fat is isolated from a classically improved and genetically modified strain of the microalgae *Prototheca moriformis*. The fat is composed primarily of triglycerides (> 98%), with minor levels of diglycerides and monoglycerides (< 2%). The fat contains mostly stearic (~55%) and oleic (~35%) fatty acids, with minor amounts of other fatty acids. Algal structuring fat is solid at room temperature with a melting point of approximately 47°C and is pale yellow to wheat-yellow in color.[31] The algal structuring fat is refined, bleached, deodorized and enriched in TAG species of the saturate-unsaturate-saturate (Sat-Unsat-Sat) type.

Algal structuring fat has not previously been added to food. Algal structuring fat will be used as a partial replacement for dietary fats in a variety of foods.[32] Algal structuring fat will only be added to foods for which a standard of identity does not exist.

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[30] Heterotrophic fermentation = cultivation without light and with the controlled addition of carbon source (*e.g.*, sugars) and other energy sources (Bumbak *et al.*, 2011).

[31] Algal structuring fat is not added to food with the intention of acting as a color. Although algal structuring fat is pale-yellow to wheat-yellow in bulk amounts, in the small amounts added to food the fat is nearly colorless. Algal structuring fat is, therefore, exempt from the definition of a color additive [FFDCA §201(t) and 21 CFR §70.3(f)] because the ingredient is not effective as a coloring agent.

[32] Baked goods, cereals and bars, snack foods, fats and oils, confectionary and chocolate-containing products.

6.2. Identification of Organism used in Ingredient Production

Algal structuring fat that is the subject of this notification is isolated from a strain of *Prototheca moriformis* that had been modified *via* classical mutagenesis and targeted genetic modification (designated by TerraVia as strain S7737). The genus *Prototheca*, which is composed of achlorophyllous[33] eukaryotic microalgae, belongs to the Trebouxiophyceae class in phylum Chlorophyta. The related *Chlorella* genus is also a member of the same class in the same phylum. Although *Prototheca* are occasionally referred to as 'colorless *Chlorella*' due their lack of chloroplasts and photosynthetic pigments and close relationship with *C. protothecoides* (aka, *Auxenochlorella protothecoides*), species within the *Prototheca* genus have several additional differentiating traits when compared with *Chlorella* spp.: (1) *Prototheca* spp. are acidophilic, (2) are thiamine auxotrophs, and (3) cannot utilize nitrate as a sole nitrogen source, but can use a variety of hydrocarbons as sole carbon sources (Running *et al.*, 2003b). Of all the heterogeneous species that comprise the *Chlorella* genus, only *C. protothecoides* shares most of these distinguishing characteristics and is recognized as being particularly closely related to *Prototheca* spp. (Conte and Pore, 1973; Running *et al.*, 2003b; Ewing *et al.*, 2014).

Prototheca are spherical or ovoid unicellular heterotrophic eukaryotes (3 – 30 µm in diameter) having double-layered cell walls that, similar to *C. protothecoides*, contain sporopollenin.[34] These achlorophyllous microalgae lack chloroplasts and buds (Lass-Florl and Mayr, 2007; Hillesheim and Bahrami, 2011; Mayorga *et al.*, 2012). Reproduction is by asexual endosporulation in which two to 16 endospores (2 – 10 µm in diameter), symmetrically or randomly arranged in the sporangia, develop until they emerge *via* rupture of the parent sporangial cell wall (DiPersio, 2001; Lass-Florl and Mayr, 2007; Hillesheim and Bahrami, 2011; Mayorga *et al.*, 2012). In the presence of adequate nutrients, reproductive cycles can occur every 5 – 6 hours with the resulting release of sporangiospores (Lass-Florl and Mayr, 2007). *P. moriformis* (including strain S7737) and its sporangiospores are highly sensitive to inactivation by heat or chemical methods (as discussed in Section 3.2 below).

Prototheca spp. are ubiquitous in the environment. Although first isolated between 1892 – 1894 from the slime fluxes (*i.e.*, sap released from tree wounds) of lime, horse chestnut and elm trees in Germany by Dr. Kruger (Krüger, 1894; Pore *et al.*, 1983; 1985), *Prototheca* spp. have since been isolated from a wide variety of sources,[35] including environmental sources such as soil, decaying plant matter, sewage, and water, as well as from human and animal sources (*e.g.*, feces, milk, sputum, and/or lesions) (Mayorga *et al.*, 2012).

The number of species assigned to the *Prototheca* genus has varied over the years as the understanding of the phylogenetic relationships between species has increased. A majority of the scientific literature currently recognizes five species in the genus: *P. wickerhamii*, *P. zopfii*, *P. stagnora*, *P. ulmea*, and *P. blaschkeae* (Roesler *et al.*, 2006; Marques *et al.*, 2008; Mayorga *et al.*, 2012). Arholdt *et al.* (2012) recognizes *P. cutis* as a sixth. *P. moriformis* has at times been considered a species of *Prototheca* (Pore, 1985; Roesler *et al.*, 2003; Ueno *et al.*, 2005; Jagielski and Lagneau, 2007), but the assignment has not been generally agreed upon as valid (Pore, 1985;

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[33] Achlorophyllous = without photosynthetic pigments.

[34] Rare but natural oxidative carotenoid polymers that occur in a few microorganisms and plants.

[35] Of 100 randomly selected strains of *Prototheca* held in the private culture collection belonging to Professor R. Scott Pore at the West Virginia University Medical School, Charleston WV), seven had been isolated from humans, 35 from animals, and 58 from the environment (Shahan and Pore, 1991; Running *et al.*, 2003a).

Roesler *et al.*, 2003; Ueno *et al.*, 2003; 2005; Lass-Florl and Mayr, 2007; Marques *et al.*, 2008; Mayorga *et al.*, 2012). Recent molecular phylogenetic data shows a strong affinity between *P. wickerhammii* and *P. moriformis* groups (Ewing *et al.*, 2014). During a recent interval in which *P. moriformis* was considered a species within *Prototheca* and *P. blaschkeae* was categorized as a variant (biotype) of *P. zopfii* (Roesler *et al.*, 2006; Mayorga *et al.*, 2012), sequence analysis of the 18S rRNA gene identified *P. blaschkeae* as a species rather than a biotype of *P. zopfii* and *P. moriformis* as a biotype of *P. zopfii* rather than its own species. Further examination of *P. zopfii* variants by 18S rRNA gene sequence analysis and cellular fatty acid composition as potential subspecies of *P. zopfii* led to the recommendation that variants 1 and 2 of the three variants analyzed be reclassified as genotypes 1 (suggested name *P. zopfii* subsp. *occulta*) and 2 (suggested name of *P. zopfii* subsp. *bovimastitogenes*), respectively of *P. zopfii* (Roesler *et al.*, 2006; Lass-Florl and Mayr, 2007).

During the study by Ueno *et al.* (2003), *P. moriformis* ATCC[36] 50081 (aka RSP-1216; isolated in 1983 by R.S. Pore from cheese factory wastewater Costa Rica[37]) was found to group closely, not with either of the genotypes, but with a central *P. zopfii* clade[38] which included *P. zopfii* var. *hydrocarbonea* ATCC 30253 (aka 48-Y; isolated by J.D. Walker in 1973 from Colgate Creek sediment, Baltimore Harbor, Chesapeake Bay, Maryland[39]) and *P. zopfii* UTEX[40] 178 (aka SAG[41] 263-1, isolated before 1912 from an unknown source[42] by M. W. Beijerinck). The authors recommended further study to determine whether this clade comprises a third subspecies (Ueno *et al.*, 2003; Roesler *et al.*, 2006). More recent taxonomic analysis clearly differentiates the *Prototheca* species into five distinct clades, which are composed of: (A) *A. protothecoides*; (B) A single *Prototheca wickerhamii* isolate; (C) A mixture of *P. wickerhamii* and *P. moriformis* isolates (including UTEX 1435, the wild type strain pertinent to this dossier); (D) *P. ulmea* and *P. stagnora*; and (E) primarily *P. zopfii* isolates with *P. moriformis*, as well as *P. kruegani* and *P. blaschkeae* (Ewing *et al.*, 2014). The authors, who examined a larger number of *Prototheca* isolates than had been included in most of the earlier studies, concluded that strains of *P. moriformis, P. wickerhamii*, and *P. stagnora* are part of paraphyletic groups that still require additional resolution.

Of the currently recognized species of *Prototheca, P. zopfii, P. wickerhamii, and P. blaschkeae*, each contain strains that have been documented as opportunistic pathogens causing rare infections (*i.e.*, protothecosis) in humans and domestic and wild animals. *P. wickerhamii* is most often associated with the rare infections reported in humans. In a typical year, two to five cases of infection are reported globally; from 1964 – 2012, a total of 160 cases of human protothecosis have been reported (Todd *et al.*, 2012). Infection in humans is often associated with traumatic introduction into the skin and underlying tissues, and systemic infection is usually associated with severe immunosuppression or underlying disease (Lass-Florl and Mayr, 2007; Mayorga *et al.*, 2012). *P. zopfii* genotype 2 is most often associated with infection in animals,

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[36] ATTC = *American Type Culture Collection*

[37] <http://www.atcc.org/Products/All/50081.aspx>; site last accessed January 28, 2014.

[38] Clade = a group of biological taxa (as species) that includes all descendants of one common ancestor.

[39] <http://www.atcc.org/Products/All/30253.aspx>; site last accessed January 28, 2014.

[40] UTEX = University of Texas Culture Collection of Algae, Austin, Texas.

[41] SAG = Sammlung von Algenkulturen Göttingen, Albrecht-von-Haller-Institute for Plant Science, University of Göttingen, Göttingen, Germany.

[42] <http://sagdb.uni-goettingen.de/detailedList.php?str_number=263-1>; site last accessed January 28, 2014.

primarily bovine mastitis in cows and occasionally infections in dogs, but is rarely associated with disease in humans. Nearly all *P. blaschkeae* strains have been isolated from swine, although the first isolate of the species originated from a case of human onychomycosis[43] and the strains are now also associated with bovine mastitis (Roesler *et al.*, 2006; Marques *et al.*, 2008). Bovine protothecal mastitis and indeed, mastitis caused by environmental microorganisms in general, is primarily a disease of poor hygiene practices and dairy management rather than due to presence or absence of the microorganism, as *Prototheca* spp. have been identified as present in the environments of dairy cattle with and without protothecal mastitis, in the environments of dairy farms with no prior history of mastitis caused by this agent (Anderson and Walker, 1988; Pore and Shahan, 1988; Enders and Weber, 1993; Corbellini *et al.*, 2001; Janosi *et al.*, 2001).

6.2.1. Wild-type strain *Prototheca moriformis* Krüger

TerraVia obtained wild-type strain *P. moriformis* Krüger UTEX 1435[44] from the University of Texas, Culture Collection of Algae located in Austin, Texas, where it had been deposited by W.B. Cooke in 1966. After undergoing clonal purification to ensure the culture was axenic (free of any contaminating organisms), UTEX 1435 was assigned the TerraVia internal strain number 'S376'. Other deposits of this strain[45] include the ATCC 16525[46] accession held at the American Type Culture Collection (ATCC, Manassas, Virginia); Hopkins IV 7.3.2.1[17] assigned by C.B. van Niel at the Hopkins Marine Station, Stanford University (Pacific Grove, California) (Sudman and Kaplan, 1973); and the SAG 263-7[47] accession deposited by M.B. Allen held in the Sammlung von Algenkulturen Göttingen (SAG) (Albrecht-von-Haller-Institute for Plant Science, University of Göttingen, Göttingen, Germany). It is generally understood that the original stock of UTEX 1435 was isolated prior to 1894 by W. Krüger from the sap of a lime tree near Jena, Germany and a horse chestnut tree in the botanical gardens at Halle an der Saale in Germany, with the strain most likely originally named *Prototheca zopfii* Krüger[48] (Krüger, 1894) and designated Pr-9 (*Prototheca* 9th strain) when deposited in the collection at the Microbiology Department, Delft Technical School, Delft, The Netherlands. The strain was later provided to C.B. van Niel and deposited in the collection at Hopkins Marine Station (Stanford University, Pacific Grove, CA) where the accession was designated Hopkins IV 7.3.2.1, before being given by van Niel to W.B. Cooke who deposited the strain in UTEX (where the accession was designated *P. moriformis* Krüger UTEX 1435) and in the ATCC (where the accession was designated *P. moriformis* Krüger ATCC 16252). The accession deposited into the SAG by M.B. Allen (designated *P. zopfii* Krüger SAG 263-7) had also been transferred by C.B. van Niel from the Hopkins collection. Recent work however, by Ewing *et al.* (2014), indicates that the SAG accession is distinct from UTEX 1435.

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[43] 'Fungal' infection of the nail and/or nail bed.

[44] <http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=3871>; site last accessed January 9, 2014.

[45] Although the CDC B-1444 accession deposited into the Center for Disease Control (CDC, Atlanta, Georgia) and the NRRL Y-6865 accession held by the Agriculture Research Service (ARS) Culture Collection (Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois) had been assigned to *P. moriformis* ATCC 16525 as identical strains (Sudman and Kaplan, 1973), they were later determined to be strains of *P. wickerhamii* (Pore, 1985).

[46] <http://www.atcc.org/Products/All/16525.aspx >; site last accessed January 9, 2014.

[47] <http://sagdb.uni-goettingen.de/detailedList.php?str_number=263-7>; site last accessed January 9, 2014.

[48] During this same time period, a second distinct strain of *Prototheca* was isolated from the slime flux of an elm tree at Halle an der Saale, Germany, and named *Prototheca moriformis* Krüger (Krüger, 1894).

P. moriformis UTEX 1435 is not the type strain for either *P. moriformis*[49] or *P. zopfii*.[50] While the ATCC preserves *P. moriformis* strain ATCC 16525 under Biosafety Level (BSL) 1 conditions,[17] indicating that the microorganism is not recognized to cause disease in immunocompetent adult humans, the SAG preserves identical accession SAG 263-7 under BSL 2 conditions,[18] which indicates that the microorganism is rarely associated with human disease and of low concern to laboratory staff.

6.2.2. Modified *Prototheca moriformis*

Mutagenesis of the wild type *P. moriformis* Krüger strain (designated by TerraVia as strain S376) was performed to improve yield and productivity of oils the microorganism already produced, as well as to alter oil composition. The steps involved in generating the classically and genetically modified strain of *P. moriformis* (S7737) used for manufacture of algal structuring fat from the wild-type *P. moriformis* Krüger strain are outlined in Table 7 and described in more detail in the text that follows.

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[49] No type strain for *P. moriformis* is recognized (Pore, 1985).

[50] *P. zopfii* Krüger SAG 263-4 (aka, UTEX 327; formerly *Prototheca portoricensis* var. *trispora* Ashford, Ciferri et Dalmau), originally isolated from a human case of enteropathia by B. K. Ashford before 1930, is the *P. zopfii* type strain (Roesler *et al.*, 2003).

Table 7. Generation of *P. moriformis* strain S7737 from wild-type *P. moriformis* Krüger strain

Strain	Origin/Modification
UTEX 1435	Original wild-type *P. moriformis* Krüger strain deposited at University of Texas, Austin TX.
S376	TerraVia internal strain designation following clonal purification of UTEX 1435.
S5100	Classical modification: S376 treated with chemical mutagens and ultraviolet light, clone screened for increased TAG accumulation, productivity and yield of oil on carbon substrate, as well as increased oleic acid content.
S5780	Genetic modification: S5100 was transformed with a cassette targeting a single allele [of an endogenous *P. moriformis* gene] to increase production of stearic acid (C18:0) and SOS content and the *A. thaliana ThiC* gene as a selectable marker.
S6573	Genetic modification: S5780 was transformed with a cassette targeting the neutral locus and comprised of the *Garcinia mangostana* gene for further increased levels of stearate and SOS content and the *Saccharomyces cerevisiae* sucrose invertase (SUC2) encoding a secreted invertase as a selectable marker.
S7737	Genetic modification: S6573 was transformed with a cassette targeting a single allele of the endogenous gene for additional increase in stearate levels along with reduction in palmitate. The cassette was comprised of a hairpin RNAi targeting endogenous [gene] expression to lower linoleic fatty acids along with expression of the *Brassica napus* gene, to reduce tri-saturated fatty acids and the gene encoding secreted melibiase (*MEL1*) from *S. carlsbergensis* as a selectable marker.

UTEX = University of Texas, TAG = triacylglycerol, [Nomenclature of the integrated genes] *SUC2* = *Saccharomyces cerevisiae* sucrose invertase gene.

P. moriformis S376 is a unicellular alga of microscopic size (10 – 12 μm). The strain has a significantly reduced plastid genome compared with *C. protothecoides*, lacks chlorophyll, and is an obligate heterotroph that can metabolize a variety of C6 sugars (*e.g.*, glucose, fructose, galactose, as well as glycerol, but not sucrose) in support of growth. Culture temperatures for growth range from 21.5 – 35.5°C and the strain tolerates pH ranges of 4 – 7. The wild-type *P. moriformis* Krüger strain initially underwent clonal purification to ensure the culture was axenic (free of any contaminating organisms). To improve the lipid content *per* cell, overall lipid production (g/l/day) and the yield of oil on carbon substrate, S376 was subjected to several rounds of classical mutagenesis involving the chemical mutagens *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and ethyl methanesulfonate (EMS) as well as ultraviolet light. The strains resulting from this classical improvement regime were screened for increased TAG (*i.e.*, triglyceride) accumulation, productivity and yield of oil on carbon substrate. A particularly promising classically modified strain producing increased levels of oleic acid received the TerraVia internal designation 'S5100'. *P. moriformis* strain S5100 is unable to utilize sucrose as a substrate, consistent with the wild-type *P. moriformis* strain S376 and its other derivatives.

The strain producing the algal structuring fat (S7737) is the result of three separate transformation events. The first transformation event was carried out in strain S5100 to generate strain S5780. Strain S5100 was transformed with integrative vector pSZ2624, which targets the disruption of a single allele of the endogenous [gene]. Disruption of a single allele of the endogenous gene results in an increase in C18:0 fatty acids. As part of the gene disruption cassette, two additional genes were introduced:

(1) The *P. moriformis* gene encoding the protein, which serves to increase carbon chain lengths in the triglyceride oil beyond C16:0.

(2) The 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase gene (*ThiC*) from *Arabidopsis thaliana* which allows the strain to grow in the absence of thiamine and serves as a selectable marker.

pSZ2624 was linearized prior to transformation into *P. moriformis*, such that the vector backbone was physically liberated from the actual integrating DNA in order to increase the efficiency of targeted integration events. The integrating portion of pSZ2624 targets the locus and can be written as follows:

Proceeding from 5' to 3', the endogenous promoter drives the expression of *P. moriformis* gene which is fused to the endogenous transit peptide , for targeting to the plastid.[51] This is followed by the *Chlorella vulgaris* 3' UTR which provides message stability and transcriptional termination. The *C. protothecoides* promoter drives expression of the *A. thaliana ThiC* gene which allows the strain to grow in the absence of exogenously supplied thiamine. The *C. protothecoides* 3' UTR provides mRNA stability and transcriptional termination. Strain S5780 was selected from among several similarly transformed strains due to its superior fatty acid profile (high stearic acid content, > 25%) and higher lipid productivity.

Strain S6573 was developed from strain S5780 by transformation with integrative vector pSZ3204 which targets integration at the neutral locus. As part of the integrative cassette, two additional genes were introduced:

(1) The *Saccharomyces cerevisiae SUC2* gene encoding for sucrose invertase which serves as a second selectable marker and allows the strain to grow on sucrose as a carbon source.
(2) The *Garcinia mangostana* thioesterase results in a further increase in stearate levels in the resulting fat.

pSZ3204 was linearized prior to transformation into S5780, such that the vector backbone was physically liberated from the actual integrating DNA in order to increase the efficiency of targeted integration events. The integrating portion of pSZ3204 targets the neutral locus and can be written as follows:

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[51] Plastid = Double membrane organelles, generally involved in either the manufacture or storage of food in algae and other plants; http://www2.mcdaniel.edu/Biology/botf99/cellstructure/plastids.html; site last visited February 19, 2016.

Proceeding from the 5' to 3' end, the *Chlamydomonas reinhardtii* [redacted] promoter drives expression of *S. cerevisiae SUC2* followed by the *Chlorella vulgaris* [redacted] 3' UTR which provides message stability and transcriptional termination. The *P. moriformis* [redacted] promoter drives expression of the *G. mangostana* [redacted] gene which is again followed by [redacted] 3' UTR to provide mRNA stability and transcriptional termination. Strain S6573 was selected from among several similarly transformed strains based upon its superior fatty acid profile (higher stearic acid content > 55%) and higher fat productivity.

Finally, strain S7737 was developed from strain S6573 by transformation with integrative vector pSZ4164 which targets integration at the [redacted] locus to increase stearate levels while reducing palmitate. As part of the integrative cassette, three additional genes were introduced:

(1) The *Saccharomyces carlsbergensis MEL1* gene encoding the melibiase enzyme which serves as a third selectable marker and allows the strain to grow on melibiose as a carbon source.
(2) The *Brassica napus* [redacted] transferase [redacted] gene which increases the level of C18:0 fatty acids at the *sn*-2 position on the glycerol backbone, thereby increasing the level of Stearate-Oleate-Stearate (SOS) triglyceride species present in the final fat and decreasing the level of tri-saturated triacylglycerol species, particularly Stearate-Stearate-Stearate (SSS).
(3) The [redacted] hairpin RNAi cassette decreases levels of C18:2 fatty acids through the RNAi-mediated silencing of the endogenous [redacted] thereby resulting in a decrease in triglyceride species of the Stearate-Linoleic-Stearate (SLS) type.

pSZ4164 was linearized prior to transformation into S6573, such that the vector backbone was physically liberated from the actual integrating DNA in order to increase the efficiency of targeted integration events. The integrating portion of pSZ4164 targets the [redacted] locus and can be written as follows:

[redacted]

[redacted]

The integrative cassette is in the reverse orientation relative to the endogenous [redacted] gene; proceeding from 5' to 3' within the cassette, the *P. moriformis* [redacted] transporter [redacted] promoter drives expression of the *S. carlsbergensis MEL1* gene followed by the *P. moriformis* [redacted] kinase [redacted] 3' UTR which provides mRNA stability and transcriptional termination. The *P. moriformis* [redacted] transporter [redacted] promoter drives expression of the *B. napus* [redacted] transferase [redacted] gene, termed [redacted] to indicate the original isolated isoform, followed by the *C. vulgaris* [redacted] 3' UTR which provides mRNA stability and transcriptional termination. Lastly, the *C. reinhardtii* [redacted] promoter drives expression of the *P. moriformis* specific [redacted] hairpin RNAi cassette [redacted], again followed by the *C. vulgaris* [redacted] 3' UTR which provides mRNA stability and transcriptional termination. Strain S7737 was selected from among several similarly transformed strains based upon a high stearic acid (>55%) and low linoleic acid (< 2%) content and superior triglyceride (> 75% Sat-Unsat-Sat; > 75% Sat-O-Sat and < 6% Sat-Sat-Sat) profiles.

6.3. History of use

Microalgae, such as *Chlorella vulgaris* and *Chlorella pyrenoidosa*, have been an accepted part of the human diet for hundreds of years and wild stocks of various microalgae are harvested as current food sources in many regions of the world (Kay, 1991; Ravishankar, *et al.*,2006). However, there is no historical documentation of *Prototheca* spp. being among those intentionally consumed by humans prior to 1958, although instances of unintentional consumption of *Prototheca* spp. by humans and animals have been reported (Pore *et al.*, 1983; Pore and Shahan, 1988; Jagielski and Lagneau, 2007).

A high oleic acid oil produced from an engineered strain of *P. moriformis* was recently evaluated for safety (Szabo *et al.*, 2014), determined GRAS by an Expert Panel and received a "no objection" letter when notified to the FDA as GRAS Notification (GRN) 000527 (FDA, 2015b).

6.4. Current uses

Strains of the genus *Prototheca* have several yeast-like properties, and have been surveyed along with oleaginous yeasts to determine growth conditions that produce fatty acids appropriate for biodiesel feedstock[52] (Sitepu *et al.*, 2013).

A number of *P. moriformis* strains (ATCC 75669,[53] BTR[54] 1080, BTR 1181, UTEX 288,[55] UTEX 1439[56]) have been evaluated for their ability to produce and accumulate, extracellularly, large quantities of ascorbic acid (vitamin C) for subsequent use in human and animal foods (Running, 1999; 2002; 2003b). These five strains were selected from a total of 43 strains of *Prototheca* screened: *P. moriformis* (five strains), *P. stagnora* (1 strain), *P. ulmae* (2 strains), *P. wickerhamii* (1 strain), and *P. zopfii* (34 strains) and 9 strains of *C. protothecoides* that had originally been considered (Running *et al.*, 2002). Based on performance, wild-type strain *P. moriformis* ATCC 75669 (BTR 1385) and its classically derived mutant strains were found to be effective at the accumulation of extracellular ascorbic acid.

Although electronic searches of the European regulatory website[57] have not revealed any documentation of *P. moriformis* strains in current use in human or animal foods, a scientific publication on the safety of a genetically engineered strain of *P. moriformis* was located in the literature (Szabo *et al.*, 2014) and a notification to FDA on the GRAS status of a high oleic acid cooking oil was found on the FDA website, with the FDA providing a "no objection" letter to the GRAS status of the high oleic oil derived from a genetically engineered strain of *P. moriformis*.[58] The food uses stated in the GRAS include: Baked goods, cereals and bars, snack foods, fats and oils, confectionaries and chocolate-containing products.

In addition, several related microalgal species have a history of consumption by humans or have recently been accepted as safe for human consumption including, but not limited to, members

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[52] Requirements for high quality feed stocks include the efficient production of specific lipid content and profiles.

[53] Strain no longer held by ATCC.

[54] BTR = private collection Bio-Technical Resources, Manitowoc, Wisconsin.

[55] UTEX 288 (aka, SAG 263-2 and CCAP 263-2), originally isolated from the slime flux emanating from a linden tree wound is categorized as a BSL 2 organism.

[56] Strain no longer held by UTEX.

[57] European Food Safety Authority.

[58] http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=527; site last accessed November 23, 2015.

of the same class (Trebouxiophyceae) and fairly distantly related *Chlorella* species (*C. vulgaris* and *C. pyrenoidosa*), which have been and are currently used as foods and ingredients in dietary supplements in the U.S. and in the EU (Nature's Balance, 2010; Dietary Supplement Label Database, 2015a; 2015b; Vitality Medical, 2016). Also in the U.S., algal oil from the closely related microalga *C. protothecoides* S106 holds GRAS status (GRAS Notification 000384)[59] as a dietary oil and food ingredient (~60% oleic, ~15% palmitic and ~10% linoleic acid) for use in a variety of human foods.

C. protothecoides S106 flour with 40 – 70% lipid (algal flour), also called AlgaVia™ Whole Algal Flour (WAF), holds GRAS status (GRN 000469)[60] as a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening in a variety of food products, including baked goods, beverages, and dairy and egg products. From the same *C. protothecoides* S106 strain, a high (40 – 75%) protein powder called AlgaVia™ Whole Algal Protein (WAP) has been determined GRAS (GRN 000519)[61] for use as a source of dietary protein in a wide variety of food products.

6.5. Proposed use or uses

Algal structuring fat is a pale yellow to wheat yellow-colored,[62] refined, bleached, and deodorized algal fat containing primarily stearic and oleic fatty acids, isolated from a modified strain of *P. moriformis* that will be used to replace a portion of the dietary fats or oils in baked goods, confectionaries, and chocolate-containing products. The levels of intended use range from 10,000 ppm[63] in a variety of foods including cookies and breads to 250,000 ppm in vegetable shortening and powdered cream substitute.

6.6. Mechanisms of action

Algal structuring fat will replace a portion of the dietary fats or oils in a variety of conventional foods. As a replacement source of macronutrients and energy, the lipids (mainly stearic and oleic fatty acids) found in the algal structuring fat will be digested through the same normal physiological processes by which other plant-derived oils common to the human diet are digested and utilized.

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[59] <http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=384>; site last accessed January 12, 2016.

[60] <http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=469>; site last accessed January 12, 2016.

[61] <http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm449793.htm>; site last accessed November 23, 2015.

[62] The algal structuring fat is not added to food with the intention of acting as a color. Although the algal structuring fat is a pale yellow to wheat yellow in bulk amounts, in the small amounts added to food the color fades to near colorlessness. Under the conditions of maximum usage in foods (up to 250,000 ppm or 25% in shortening, vegetable, and cream substitute, powdered), the color of the food to which the algal structuring fat is added is not altered. The algal structuring fat is, therefore, exempt from the definition of a color additive [FFDCA §201(t) and 21 CFR§70.3(f)] because the ingredient is not effective as a coloring agent.

[63] ppm = parts *per* million.

6.7. Regulatory Status

The algal structuring fat isolated from a modified strain of *P. moriformis* has not been approved for use in food by FDA[64], FEMA[65], or USDA.[66] Although *Prototheca* spp. in a dairy herd environment holds an entry in the FDA Poisonous Plant Database,[67] the entry is generic and has not been updated since 1988. A high oleic algal oil ingredient produced with a related *P. moriformis* strain that underwent classical mutagenesis and targeted genetic modification was determined GRAS, notified to FDA of the GRAS status, and received a "no objection" letter from FDA (GRN 000527).[68]

Algal oil from the related microalga *C. protothecoides* S106 holds GRAS status (GRN 000384)[69] as a food oil to be used in a variety of foods excluding meat and poultry products. In addition, *C. protothecoides* S106 flour with 40 – 70% lipid, called AlgaVia™ Whole Algal Flour (WAF), also holds GRAS status (GRN 000469)[70] as a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening in baked goods, beverages, dairy and egg products, sauces, gravies, margarines, salad dressings, and soups. Although a determination has not been made for WAF in Europe, WAF derived from the closely related strain of *C. protothecoides* is not a novel food according to the Novel Food Regulation (EC) No. 258-97, but a traditional food ingredient (TNO, 2012). The EU's Scientific Committee on Food does not have an established Acceptable Daily Intake (ADI) for WAF in humans. From the same *C. protothecoides* S106 strain, a high (40 – 75%) protein powder called AlgaVia™ Whole Algal Protein (WAP) has been determined GRAS (GRN 000519)[71] for use as a source of dietary protein in a variety of food products.

6.8. Description, Specifications and Manufacturing Process

6.8.1. Description and Specifications

The algal structuring fat is a pale yellow to wheat yellow-colored, refined, bleached and deodorized fat isolated from an engineered strain of the microalga *P. moriformis*. Typical physical and chemical properties of the high-oleic algal oil are provided in Table 8. The specifications for the high-oleic algal oil are provided in Table 9. The neutral-flavored oil is almost exclusively in the form of triglycerides (≥ 95%) with small amounts of diglycerides (< 5%) and trace amounts of monoglycerides (< 0.5%). Small quantities of free fatty acids (≤ 0.1%) and polar compounds (< 4%) are also present. Non-saponifiable matter comprises ≤ 1% of the oil, with moisture being ≤ 0.1%. In the three lots presented in Table 9 and Table 10, stearic acid was the predominant fatty

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[64] FDA = United States Food and Drug Administration.

[65] FEMA = Flavor Extract Manufactures Associations.

[66] USDA = United States Department of Agriculture.

[67] <http://www.accessdata.fda.gov/scripts/Plantox/Detail.CFM?ID=945>; site last accessed December 22, 2015.

[68] Agency Response Letter GRAS Notice No. GRN 000527; <http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm469231.htm>; site last accessed December 22, 2015.

[69] <http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=384>; site last accessed December 22, 2015.

[70] <http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=469>; site last accessed December 22, 2015.

[71] <http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm449793.htm>; site last accessed November 23, 2015.

acid (≥ 50 Area % Total Fatty Acid (TFA)) followed by oleic (~30 Area % TFA) and palmitic acids (~2 Area % TFA) and lesser amounts of other fatty acids.

Table 8. Typical physical and chemical properties of the algal structuring fat

Characteristic	Value
Synonyms	Algal structured fat; SOS algal fat
Appearance	Pale yellow to wheat yellow solid
Smoke point	201 ± 4°C
Flash point	227 ± 5°C
Neutral oil	99 ± 0.3 %
Fatty acid profile	
C18:0	≥ 50 Area %
C18:1	≥ 30 Area %
C18:2	≤ 2 Area %
C18:3 *alpha*	≤ 0.8 Area %
Total saturates	≥ 50 Area %
Free fatty acids	≤ 1.0 %
Moisture content*	≤ 250 ppm
Unsaponifiable matter	≤ 2 %
Insoluble matter	≤ 0.1 %
Triglycerides	≥ 95.0 %
Diglycerides	≤ 5.0 %
Monoglycerides	≤ 0.5 %
Total polar compounds	< 5.0 %

C18:0 = Stearic acid; C18:1 = Oleic acid; C18:2 = Linoleic acid; C18:3 *alpha* = *Alpha*-Linolenic acid; SOS = stearic-oleic-stearic; *Karl Fisher moisture, determined by AOCS Ca 2e-84 and stated in parts per million (ppm)

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Table 9. Specifications for the algal structuring fat

Parameter	Method	Specification	Batch Analysis Results (n=3) Range	Batch Analysis Results (n=3) Average
Appearance	Visual inspection	Pale yellow to wheat yellow solid	Conforms	Conforms
Odor	Olfactory inspection	Slight	Conforms	Conforms
Fatty acid profile	C-M-00036-000 Rev 0[a]			
Palmitic acid (C16:0)	C-M-00036-000 Rev 0[a]	≥ 2 Area %	4.15 – 4.35 Area %	4.24 Area %
Stearic acid (C18:0)	C-M-00036-000 Rev 0[a]	≥ 50 Area %	54.6 – 55.5 Area %	55.1 Area %
Palmitic + Stearic (C16:0 + C18:0)	C-M-00036-000 Rev 0[a]	≥ 55 Area %	58.9 – 59.7 Area %	59.3 Area %
Oleic acid (C18:1)	C-M-00036-000 Rev 0[a]	≥ 30 Area %	34.8 – 35.0 Area %	34.9 Area %
Linoleic acid (C18:2)	C-M-00036-000 Rev 0[a]	≤ 2 Area %	1.26 – 1.31 Area %	1.29 Area %
Alpha-Linolenic acid (C18:3 *alpha*)	C-M-00036-000 Rev 0[a]	≤ 0.8 Area %	0.24 – 0.28 Area %	0.27 Area %
Total Saturated Fat	C-M-00036-000 Rev 0[a]	≥ 50 Area %	61.4 – 62.0 Area %	61.7 Area %
Free Fatty Acids	AOCS Ca 5a-40	≤ 1%	0.38 – 0.58%	0.48%
Unsaponifiable Matter	AOCS Ca 6a-40	≤ 2%	0.63 – 1.07%	0.84%
Peroxide Value	AOCS Cd 8-53	≤ 2 meq/kg	BLD[c]	BLD[c]
p-Anisidine Value	ISO 6885	≤ 2	0.3 – 0.38	0.35
Elements by ICP				
Lead	AOCS Ca 17-01	< 0.2 ppm	< 0.20 ppm	< 0.20 ppm
Arsenic	AOCS Ca 17-01	< 0.2 ppm	< 0.20 ppm	< 0.20 ppm
Mercury	AOCS Ca 17-01	< 0.2 ppm	< 0.20 ppm	< 0.20 ppm
Cadmium	AOCS Ca 17-01	< 0.1 ppm	< 0.03 ppm	< 0.03 ppm
Phosphorus	AOCS Ca 20-99	≤ 0.5 ppm	< 0.20 ppm	< 0.20 ppm
Sulfur	AOCS Ca 17-01	≤ 1 ppm	< 0.5 ppm	< 0.5 ppm

[a] C-M-00036-000 Rev 0, Total Deliverable Fatty Acid Methyl Ester Content and Profiles of Algal Oil Samples by Direct Transesterification (internal method; as provided in GRAS dossier (TerraVia, 2016).

[b] C-M-00118-000 Rev 1, Karl Fischer Moisture Determination of Oil (internal method; as provided in GRAS dossier (TerraVia, 2016)).

[c] BLD = Values below the limit of detection = 0.1 meq/kg.

AOCS = American Oil Chemists' Society; ISO = International Standards Organization; meq = milliequivalents; ppm = parts *per* million.

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Table 10. Fatty acid composition (as a percentage of the total fatty acid content) for three lots of the algal structured fat compared with conventional structured fat

Fatty Acids (Area %)	Lot # RBD734	Lot # RBD735	Lot # RBD736	Mean (n = 3)	Kokum butter [b]	Shea Stearin [c]	Oil, Shea nut [d]	Oil, Cocoa butter [d]
C8:0	ND	ND	ND	ND	ND	ND	0.20	0.00
C10:0 (Capric)	0.05	0.04	0.08	0.06	0.01	0.02	0.20	0.00
C11:0	ND	ND	ND	ND	ND	ND	NR	NR
C12:0 (Lauric)	0.23	0.24	0.35	0.27	0.05	0.17	1.30	0.00
C12:1	ND	ND	ND	ND	ND	ND	NR	NR
C13:0	ND	ND	ND	ND	ND	ND	NR	NR
C14:0 (Myristic)	0.52	0.54	0.61	0.56	0.04	0.09	0.10	0.10
C14:1, *cis*-9 (Myristoleic)	ND	ND	ND	ND	ND	ND	NR	NR
C15:0 (Pentadecanoic)	0.01	0.02	0.01	0.01	0.01	ND	NR	NR
C16:0 (Palmitic)	4.15	4.22	4.35	4.24	2.05	3.16	4.40	25.40
C16:1, *cis*-7	0.07	0.07	0.07	0.07	0.02	ND	0.10	0.20
C16:1, *cis*-9 (Palmitoleic)	0.06	0.07	0.07	0.07	0.01	0.02	NR	NR
C17:0 (Heptadecanoic)	0.09	0.09	0.09	0.09	0.11	0.10	NR	NR
C17:1, *cis*-9	0.05	0.01	0.05	0.04	ND	ND	NR	NR
C18:0 (Stearic)	55.53	55.12	54.59	55.08	57.47	57.73	38.80	33.20
C18:1, *cis*-9 (Oleic)	34.83	34.98	35.02	34.94	38.18	33.05	43.50	32.60
C18:2, *cis*-9,12 (Linoleic)	1.26	1.29	1.31	1.29	0.83	2.85	4.90	2.80
C18:3 alpha	0.24	0.28	0.28	0.27	0.08	0.05	0.30	0.10
C20:0 (Arachidic)	1.39	1.36	1.34	1.36	0.47	1.74	NR	NR
C20:1	0.10	0.10	0.10	0.10	0.18	0.11	0.00	0.00
C20:2	0.02	0.03	0.02	0.02	ND	ND	NR	NR
C20:3n3	0.01	0.01	ND	0.01 [a]	ND	ND	NR	NR
C21:0	ND	ND	ND	ND	ND	ND	NR	NR
C22:0 (Behenic)	0.14	0.14	0.14	0.14	0.03	0.16	NR	NR
C22:1, *cis*-13 (Erucic)	ND	ND	ND	ND	ND	ND	0.00	0.00
C22:2n6	0.08	0.10	0.09	0.09	ND	0.04	NR	NR
C23:0 (Tricosanoic)	ND	ND	ND	ND	ND	ND	NR	NR
C24:0 (Lignoceric)	0.07	0.08	0.06	0.07	ND	0.11	NR	NR
Total Fatty Acid	98.90	98.77	98.63	98.77	99.54	99.40	93.80	94.40
TAG Profiles								
Saturate-Saturate-Saturate (Sat-Sat-Sat)	4.79	4.52	4.35	4.55	0.82	2.40	NA	NA
Saturate-Unsaturate-Saturate (Sat-Unsat-Sat)	79.38	80.42	79.93	79.91	83.42	88.80	NA	NA
Saturate-Oleic-Saturate (Sat-O-Sat)	76.44	77.44	76.75	76.88	81.66	82.70	NA	NA
Melting Point (°C)	46.8	46.9	46.1	46.6	37.0 – 40.0			

[a] $n = 2$.

[b] Obtained from Essential Wholesale & Labs and refined, bleached, and deodorized.

[c] Obtained from Archer Daniel Midlands (ADM) Corporation.

[d] USDA National Nutrient Database for Standard Reference, Release 27 (<http://ndb.nal.usda.gov/ndb/search/list>; site accessed June 26, 2015).

n = number; NA = Not analyzed; ND = Analyzed, but not detected; NR = Not reported; TAG = Triacylglyceride.

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Pheophorbides are naturally occurring degradation products of chlorophyll that form during 'de-greening' events[72] (Jassby, 1988). Ingestion of pheophorbide-A (Pheide A) is known to induce photosensitive dermatitis in humans (Jitsukawa *et al.*, 1984; Jassby, 1988). Limits for existing and potential Pheide A concentrations in algal preparations (0.8 mg/g and < 1.2 mg/g, respectively) have been established by the Japanese Public Health Ministry (Becker, 1994). When high-oleic algal oil from a different genetically engineered strain of *P. moriformis* was assayed *via* high-performance liquid chromatography (HPLC) with fluorescence detection, no Pheide A was detected (Szabo *et al.*, 2014; Takano *et al.*, 2014).[73] Three lots of algal structuring fat were analyzed utilizing the same HPLC methodology and it was found that the average level of Pheide A was 0.3 ppm (0.0003 mg/g) (UBE, 2015). This result falls under the limit of quantification[47] and is far below any level of concern. *P. moriformis* itself lacks chloroplasts and chlorophyll, precluding pheophorbide formation.

Algal and cyanobacterial toxins have a broad range of potencies. Ingestion of one or more of these toxins by a human can cause a range of effects from mild illness to death. Algal structuring fat was analyzed by liquid chromatography with tandem mass spectrometric detection for the common groups of algal and cyanotoxins, including 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; 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. No toxins were detected (Food GmbH Jena Analytik, 2015).[74]

6.9. Manufacturing Process

Algal structuring fat is manufactured by fermenting and harvesting cultures of the engineered *P. moriformis* strain (S7737), a strain classically improved and genetically modified from *P. moriformis* S376 (UTEX 1435). Grown under controlled fermentation conditions, the biomass is prepared by fed-batch axenic fermentation under sterile conditions at a facility compliant with current Good Manufacturing Practice (cGMP). As *per* cGMP, cell banks are prepared to verify the identity and axenic nature of the cultures used in fermentation. Throughout the fermentation process, pH, temperature, agitation, and aeration rates are controlled, as are additions of glucose or sucrose and nutrients (as provided in GRAS dossier (TerraVia, 2016)). Following completion of fermentation, the broth is inactivated by heat and concurrently concentrated in an evaporator (≥ 10 hours at 60°C) and dried (140 – 170°C, 6 – 15 seconds), after which the dried biomass is mechanically extracted to release the crude algal oil. The above inactivation step kills all cells, vegetative and endospores. Inactivation occurs at 60°C with a minimum residence time of 120 seconds. The crude oil is then refined using standard edible oil refining steps, including degumming (by precipitation with water and/or citric acid or other acid approved for use in food), bleaching (with bleaching earth under vacuum at [high temp]), and

[72] Pheophorbide levels are increased as a plant dies, as the chlorophyll molecule loses a magnesium atom and phytol residue (Jassby, 1988).

[73] Limits of detection were 0.5 ppm for pheophorbide A.

[74] Limits of detection ranged from 0.0008 – 0.1 μg/g in the fat.

deodorization (*via* heat and steam sparging under vacuum at [high temp]).[75] To ensure stability a food-grade antioxidant (as provided in GRAS dossier (TerraVia, 2016)) may be added prior to packaging, although at present the use of such an agent is not part of the standard process. Except for one substance (*i.e.*, soybean hulls),[76] all ingredients used during manufacture are safe and suitable for human use (TerraVia, 2016). Soybean hulls are a fibrous material used as an extraction aid to assist in release of the crude oil from the algal cells.

To support safe use as a press aid, the soybean hulls are assayed for pesticides and potential toxins. Milled soy bean hulls and the final refined, bleached, and deodorized high-oleic algal oil are assayed for pesticides (*i.e.*, polychlorinated biphenyls (PCBs), hexachlorobenzene, methoxychlor, mirex, aldrin, endrin, lindane, chlordane, heptachlor, heptachlor epoxide, benzene hexachloride (BHC), dieldrin, total DDT, parathion, methyl parathion, ethion, methyl trithion, malathion, ronnel, diazinon, disulfeton, and phorate) and mycotoxins (*i.e.*, < 5 ppb[77] aflatoxins, < 25 ppb zearalenone, < 1000 ppb fumonisin, < 25 ppb T-2 toxins, < 2 ppb ochratoxins, and < 500 ppb vomitoxins). No pesticides have been detected and no mycotoxins have been detected above threshold limits.

The final stages of oil manufacture include the refining steps (*i.e.*, degumming, bleaching and deodorization) which efficiently convert the crude oil into the high purity final product. A graphical depiction of the manufacturing process is presented in Figure 2. The final product, algal structuring fat, is transferred into 44 gallon drums and stored at room temperature (22 – 27ºC) for most distributors, and loaded into totes, bulk trucks and rail cars for large-scale purchase and use.

Regarding labeling concerns related to the eight major food allergens, an extraction aid used in the production of the algal structuring fat is soybean hulls. The soybean hulls used during extraction are not a cause for allergenic concern due to the refining process of the finished algal product. Two closely related high oleic algal oils (also modified from wild type strain *P. moriformis* S376) were manufactured in the same manner as the algal structuring fat of this GRAS Dossier. When submitted to the Food Allergy Research and Resource Program at the University of Nebraska (Lincoln, Nebraska) for assay, residual soy allergens were determined in the two crude clarified oils (20 and 25 ppm), but not in the refined, bleached and deodorized (finished) oils (< 2.5 ppm, limit of quantitation) (University of Nebraska, 2013). *Per* the US FDA's Food Allergen Labeling and Consumer Protection Act (FALCPA), a highly refined oil derived from one of the eight foods or food groups is not considered a major food allergen.[78]

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[75] During the refinement process, samples of the oil product (2 mL) are incubated at 28°C in media for five days alongside negative (media only) and positive controls (1000 CFU of test strain in media) to verify the absence of viable cells. Counts of test plates and negative control plates are negative; counts of positive plates range from 10^7 – 10^8 CFU.

[76] Soybean hulls are approved for use in animal feed (AAFCO, 2015).

[77] ppb = parts *per* billion.

[78] http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Allergens/ucm106890.htm#q15; site last accessed February 19, 2016.

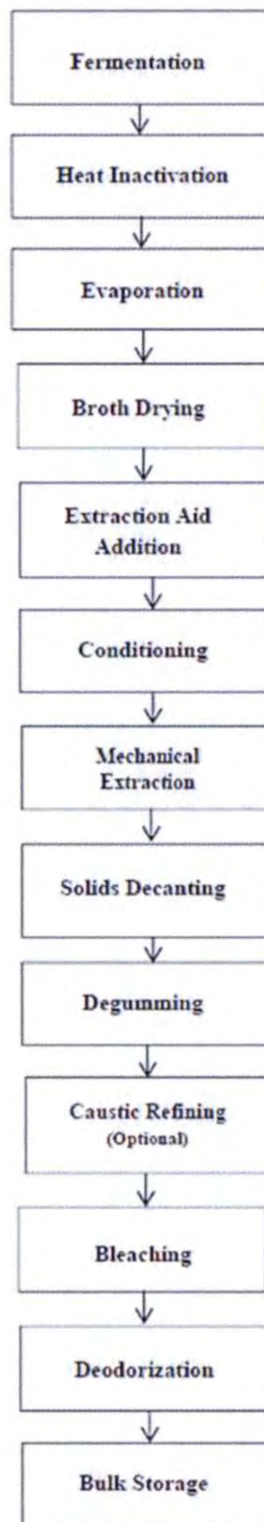

Figure 2. General production process for algal structuring fat

6.10. Stability

Whether or not an antioxidant (*i.e.*, 200 ppm tertiary butylhydroquinone) is present, algal structuring fat isolated from *P. moriformis* is stable for six months when stored at controlled room temperature,[79] 22 – 27ºC (Table 11) or elevated temperature, 40ºC (Table 12); all of the six lots had peroxide values below the allowed limit (≤ 5 meq/kg) for food grade oils, indicating a low potential for rancidity during storage. The *p*-Anisidine value (p-AV), another method of determining potential oxidation action does not significantly change during the six month stability test, either with or without added antioxidant. During the stability evaluations, the algal structuring fat was stored in 20 and 500 mL amber glass bottles.

Table 11. Stability of the algal structuring fat at room temperature

		RBD-AO-RT* (fat with antioxidant)			
Month	**PV (meq/kg)**	**p-AV**	**FFA (%)**	**OSI (110ºC)**	**Color (5.25")**
0	0.00 ± 0.00	0.75 ± 0.47	0.23 ± 0.24	52.65 ± 14.52	1.05 ± 0.3R 11.7 ± 2.6Y
1	0.34 ± 0.04	0.78 ± 0.45			
2	0.34 ± 0.07	0.77 ± 0.43			
3	0.50 ± 0.16	0.72 ± 0.35	0.26 ± 0.27		
6	0.54 ± 0.14	0.86 ± 0.43	0.30 ± 0.31		
9	0.68 ± 0.18	0.83 ± 0.37	0.32 ± 0.33		
12	0.63 ± 0.24#	0.80 ± 0.33	0.32 ± 0.33		1.8 ± 0.78R 24.6 ± 7.9Y
18	0.75 ± 0.23	0.89 ± 0.36	0.32 ± 0.34		2.3 ± 1.1R 1.1 ± 13.3Y
		RBD-AO-RT* (fat without antioxidant)			
0	0.00 ± 0.00	0.75 ± 0.45	0.24 ± 0.26	39.82 ± 13.50	1.0 ± 0.3R 11.7 ± 2.6Y
1	0.34 ± 0.07	0.80 ± 0.42			
2	0.34 ± 0.09	0.79 ± 0.45			
3	0.46 ± 0.20	0.84 ± 0.41	0.25 ± 0.27		
6	0.72 ± 0.30	0.88 ± 0.38	0.24 ± 0.26		
9	0.63 ± 0.20	0.84 ± 0.41	0.27 ± 0.28		
12	0.53 ± 0.27	0.81 ± 0.37	0.26 ± 0.27		1.4 ± 0.4R 19.6 ± 2.2Y
18	0.82 ± 0.44	0.91 ± 0.37	0.27 ± 0.27		1.6 ± 0.6R 22.7 ± 5.0Y

* Mean ± SD values for RBD lots 731, 732, 733, 734, 735, 736; FFA = Free fatty acids; OSI = Oil Stability Index; SD = Standard deviation; PV = Peroxide Value; p-AV = *p*-Anisidine Value; #Value based on five lots, as one value was below limit of detection (< 0.10).

[79] Relative humidity was not monitored or controlled because the samples used in the assays for most parameters were stored in amber glass bottles or vials with a Teflon-lined screw cap that prevented moisture transfer during the temperature treatment. In the case of the test samples used in the peroxide value and *p*-Anisidine value assays, these samples were stored in individual 20 mL amber screw cap glass vials that were filled at time zero and only opened when tested on the designated analysis date.

Table 12. Stability of the algal structuring fat at 40°C

Month	PV (meq/kg)	p-AV	FFA (%)	OSI (110°C)	Color (5.25")
		RBD-AO-40C* (fat with antioxidant)			
0	0.00 ± 0.00	0.75 ± 0.47	0.23 ± 0.24	52.65 ± 14.52	1.1 ± 0.3R 11.7 ± 2.6Y
1	0.65 ± 0.06	0.79 ± 0.45			
2	0.78 ± 0.13	0.79 ± 0.42			
3	0.79 ± 0.20	0.86 ± 0.41	0.25 ± 0.27		
6	0.80 ± 0.20	0.85 ± 0.41	0.31 ± 0.31		
9	0.71 ±0.3	0.95 ± 0.40	0.31 ± 0.32		
12	0.39 ± 0.24	0.97 ± 0.42	0.34 ± 0.36		1.4 ± 0.4R 18.6 ± 4.9Y
		RBD-AO-40C* (fat without antioxidant)			
0	0.00 ± 0.00	0.75 ± 0.45	0.24 ± 0.26	39.82 ± 13.50	1.0 ± 0.3R 11.7 ± 2.6Y
1	0.88 ± 0.50	0.85 ± 0.39			
2	1.43 ± 0.97	1.19 ± 1.04			
3	1.03 ± 0.24	0.91 ± 0.33	0.27 ± 0.28		
6	1.17 ± 0.23	0.91 ± 0.32	0.21 ± 0.24		
9	0.88 ± 0.37	1.07 ±0.37	0.29 ±0.26		
12	0.76±0.25	1.24±0.55	0.27±0.28		1.1 ± 0.4R 15.4 ± 4.3Y

* Mean ± SD values for RBD lots 731, 732, 733, 734, 735, 736; FFA = Free fatty acids; OSI = Oil Stability Index; SD = Standard deviation; PV = Peroxide Value; p-AV = *p*-Anisidine Value.

6.11. Estimated Daily Intake

The intake profile (amount and frequency) by individuals in USDA's What We Eat in America (WWEIA) Continuing Survey of Food Intakes by Individuals 2011-2012 (Dwyer *et al.*, 2003)[80] was used to calculate the Estimated Daily Intake (EDI) of algal structuring fat for individuals consuming the food groups selected for the addition of the algal structuring fat *per* this GRAS evaluation (*i.e.*, "eaters only"). The food categories to which algal structuring fat will be added includes bakery, cookie and chocolate-containing food products, and butter/margarine-like products (Table 13). Only certain foods within these categories have been selected; the individual foods selected for addition of the algal structuring fat are provided in the GRAS dossier (TerraVia, 2016).

The means and 90th percentile EDIs were calculated only for algal structuring fat intake following addition of algal structuring fat to the selected food groups. The means and 90th percentile EDIs were not calculated for current algal structuring fat intake from natural sources as no information regarding current intakes of algal structuring fat from natural sources was discovered during a comprehensive search of the published literature. Addition of algal structuring fat to the selected foods at the levels specified in the GRAS dossier (TerraVia, 2016) would provide a mean and 90th percentile algal structuring fat consumption of 3.82 and 9.10 g/day, respectively (Table 14).

[80] USDA (2014) What We Eat In America, NHANES 2011-2012, Dietary intake data fact sheet; Documentation and Data Files. USDA, Agriculture Research Service; http://www.ars.usda.gov/Services/docs.htm?docid=18354; site last accessed December 9, 2015.

All food categories designated by TerraVia have been utilized in the calculations as appropriate; however, certain categories designated by TerraVia may include foods for which a standard of identity exists. We note that an ingredient that is lawfully added to food products may be used in a "standardized" food only if it is permitted by the applicable standard of identity. TerraVia confirms that algal structuring fat will be added only to foods for which a standard of identity does not exist.

Table 13. Food groups selected for algal structuring fat supplementation*

Food Category	Maximum intended use level (ppm)
Margarine and margarine-like spreads (12)	150,000
Butter-like spreads (12)	200,000
Vegetable shortenings (12)	250,000
Nut spreads (32)	20,000
Milk products (31)	20,000
Non-dairy products (10)	250,000
Baked goods (1)	100,000
Chocolate-based sauces and syrups (43)	10,000
Baked desserts (1)	30,000
Cookies (1)	50,000
Frozen dairy desserts (20)	20,000

*The food categories correspond to those listed in 21 CFR §170.3(n). The number in parenthesis following each food category is the paragraph listing in 21 CFR §170.3(n) for that food category.
ppm = parts *per* million.

Table 14. Predicted intake of the algal structuring fat following supplementation of selected foods at the indicated levels (TerraVia, 2016) for individuals consuming selected foods

	Per User (g/day)	
Algal structuring fat intake from:	**Mean**	**90th Percentile**
Possible maximum consumption with algal structuring fat as an added ingredient to food	3.82	9.10

6.12. Absorption, Distribution, Metabolism, and Elimination (ADME)

The main lipids found in this algal structuring fat ingredient are stearic acid (approximately 55%), oleic acid (approximately 35%) and palmitic acid (approximately 4%). Fatty acids released from triacylglycerol molecules are typically degraded by β-oxidation in the mitochondria, resulting in the successive release of two-carbon acetyl coenzyme A (acetyl-CoA) molecules. Acetyl-CoA is then used in the citric acid cycle to produce reducing equivalents which are then used by the electron transport chain to produce adenosine triphosphate (ATP) for energy (Gotoh *et al.*, 2008). Algal structuring fat is expected to be digested, absorbed, metabolized and excreted through the same normal physiological processes by which other solid fats (*e.g.*, beef tallow, cocoa butter) common to the human diet are digested and utilized as macronutrients.

Stearic acid is a saturated long-chain fatty acid (18:0) that is found in milk fats, beef and other animal fats, as well as cocoa and shea butters (Sampath and Ntambi, 2005). Studies indicate that stearic acid may not be completely absorbed in the intestine, with reported absorption ranging from 68 – 98%, while most other fatty acids are consistently > 95% absorbed (Jones *et al.*, 1999;

Baer *et al.*, 2003). Javadi *et al.* (2004) reported that the addition of four *percent* (w/w[81]) stearic acid to a high-fat semi-purified diet fed to BALB/c mice for 35 days resulted in a small but significant ($p < 0.05$) decrease in metabolizable energy, as well as an increase in excreted stearic acid (in any component of excrement, including fat) as a function of energy balance. When analyzed as a *percentage* of energy intake, consumption of stearic acid resulted in a significantly greater amount of stearic acid lost in the excrement as fat ($p < 0.05$) and a significantly lower amount expended as heat, when compared to a control diet, which further indicates reduced absorption of stearic acid (Javadi *et al.*, 2004). This small decrease in absorption may be a factor in the finding that consumption of stearic acid may even lower LDL cholesterol, be neutral with respect to high-density lipoprotein (HDL) cholesterol and lower the ratio of total to HDL cholesterol, when compared to unsaturated fatty acids (Berry *et al.*, 2007; Hunter *et al.*, 2010). However, this potential decrease in absorption does not result in significant weight loss, as Volger *et al.* (2008) reported that administration of 600 mg/kg stearic acid every twelve hours for seven days to male Wistar Kyoto rats had decreased absorption and increased fecal excretion of stearic acid, but no reduction in body weight. In the same Wistar Kyoto rat study, oleic acid ingestion resulted in body weight reduction ($p < 0.05$), consistent with the reported reduction in food intake. The decrease in stearic acid absorption and consequent metabolism may simply be due to the higher solid fat content when consumed at body temperature (37°C), because fats that are solids at body temperature are less able to form micelles, a critical step in the rate of lipolysis formation and eventual fatty acid uptake (Berry and Sanders, 2005). In addition, it has been hypothesized that the structure of the triacylglycerol impacts the dietary fat metabolism, as the pancreatic lipase and lipoprotein lipase preferentially hydrolyze at the *sn*-1 and *sn*-3 positions; therefore, stearic acid at the *sn*-1 and *sn*-3 positions are absorbed to less of an extent than when at the *sn*-2 position (Berry, 2009). However, the decrease in absorption of stearic acid may not be significant in humans, as determined by Tuomasjukka *et al.* (2007), who reported a non-significant ($p > 0.05$) 87% absorption of stearic acid[82] after consumption of a meal containing an added 20 g of stearic acid, compared with a high-fat meal containing only 3.8 g stearic acid. Other studies report that stearic acid may not be completely oxidized, again reducing immediate energy production (Bessesen *et al.*, 2000; Baer *et al.*, 2003). Stearate can be metabolized by stearoyl-CoA desaturase and converted to oleate, for further lipid synthesis and oxidation to produce energy, or further elongated to form other long-chain saturated fatty acids (Sampath and Ntambi, 2005). Unlike other long-chain fatty acids, stearic acid does not raise low density lipoprotein (LDL) cholesterol or increase thrombotic-promoting actions, as evidenced by significantly reduced mean platelet volume in male volunteers provided 19.4 g/day stearic acid[83] (Kelly *et al.*, 1999).

6.13. SAFETY EVALATION

The composition of the algal structuring fat includes approximately 55% stearic acid and 35% oleic acid of the total fatty content. Stearic acid, as one of several saturated fatty acids, was initially considered detrimental to health (Tove, 1964). However, modern nutritional science has concluded that triacylglycerides containing stearic acid are neutral in its effects on blood cholesterol, as discussed in more detail below. The following safety evaluation includes an analysis

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[81] w/w=weight/weight

[82] As consumed from Salatrim, a molecule with at least one short-chain fatty acid and at least one long-chain saturated fatty acid (usually stearic acid) (Tuomasjukka *et al.*, 2007).

[83] The consumption of stearic acid during the baseline period was found to be 7.3 g/day.

of safety studies conducted to determine the effect of algal structuring fat and stearic acid on human health, specifically cardiovascular health.

6.13.1. Acute Studies

No acute toxicity studies related to *P. moriformis* or the closely related *C. protothecoides* (*A. protothecoides*) or stearic acid-rich algal fats derived from these sources were discovered in the scientific literature.

6.13.2. Short Term Repeated-Dose Studies

No short-term, repeat-dose preclinical studies of an algal structuring fat produced from a modified strain of *P. moriformis* were located in the scientific literature. However, one short-term study was conducted on a stearic acid-rich product fed to mice. Tove (1964) evaluated the toxicity of diets containing high amounts of palmitic acid (as glyceryl monopalmitate) or stearic acid (as glyceryl monostearate) when administered in the diet to weanling and adult mice (strain not stated) for three weeks. The doses were 0, 5, 10, 20, 30, 40, and 50% of the diet as glyceryl monostearate (equivalent to 0, ~7500, ~15,000, ~30,000, ~45,000, ~60,000, and ~75,000 mg/kg bw[84]/day, respectively). Decreased weight gain and mortality occurred when the weanling mice were fed glyceryl monopalmitate at 20% of the diet or greater. A diet containing 40% glyceryl palmitate was toxic to adult mice. Decreased weight gain occurred when the weanling mice were fed glyceryl monostearate at 30% of the diet or greater, but mortality (17%) did not occur until 50% of the diet was glyceryl monostearate. The primary sign of toxicity was emaciation, indicated by a decrease in adipose tissue. However, the addition of linoleic or oleic acid reversed the toxicity, proving that the toxicity was due to essential fatty acid deficiency. Diets high ($\geq$ 30% of the diet) in stearic acid resulted in decreased weight gain, which was reversed by addition of essential fatty acids.

6.14. Subchronic Studies

6.14.1. Algal structuring fat subchronic repeated-dose toxicity study

A 13-week dietary study in rats was used to evaluate the potential of algal structuring fat or kokum fat[85] to produce toxicity (Matulka *et al.*, 2016). The preclinical study followed well-established, internationally-accepted protocol guidelines, which have been utilized to evaluate the potential for toxicological effects of other algal-based food ingredients (Szabo *et al.*, 2012; 2013; 2014). 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*=10/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) and were formulated to provide the same caloric intake between groups. The test and control diets were provided to the rats *ad libitum* throughout the 13-week study. Following the treatment period, all surviving rats were fasted $\geq$ 15 hours and terminated by exsanguination.

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 the

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[84] bw=body weight

[85] Kokum fat is currently consumed as a cocoa butter equivalent in Europe and is similar in composition to algal structuring fat (Lipp and Anklam, 1998).

acclimation period, including just prior to study 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. Individual food consumption was also recorded weekly and just prior to terminal sacrifice on the same schedule as body weight measurements. 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 (Table 16 and Table 17). 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) 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 (Table 18 and Table 19). Organs and tissues from all animals were preserved in 10% neutral buffered formalin for possible future histopathological examination.[86] 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 of control and all high-dose groups and any unscheduled deaths or gross lesions from animals in any of the other dose groups were performed by a board-certified veterinary pathologist at Histo-Scientific Research Laboratories (Frederick, MD).

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), respectively. 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 administration 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

[86] Accessory genital organs (prostate and seminal vesicles), adrenals, aorta, bone (femur), bone marrow (from femur and sternum), brain (medulla/pons, cerebellar, cerebral cortex), cecum, cervix, colon, duodenum, esophagus, Harderian gland, heart, ileum with Peyer's patches, jejunum, kidneys, larynx, liver, lungs, lymph node (mandibular), lymph node (mesenteric), mammary gland, nose and nasal turbinates, ovaries, pancreas, peripheral nerve (sciatic), pharynx, pituitary, rectum, salivary glands (sublingual, submandibular, and parotid), skeletal muscle, skin, spinal cord (cervical, mid-thoracic, and lumbar), spleen, sternum, stomach, thymus, thyroid/parathyroid, trachea, urinary bladder, uterus and oviducts, and vagina.

(Figure 3 and Figure 4). Mean weekly and overall body weights and calculated 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 and calculated 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 and calculated 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.

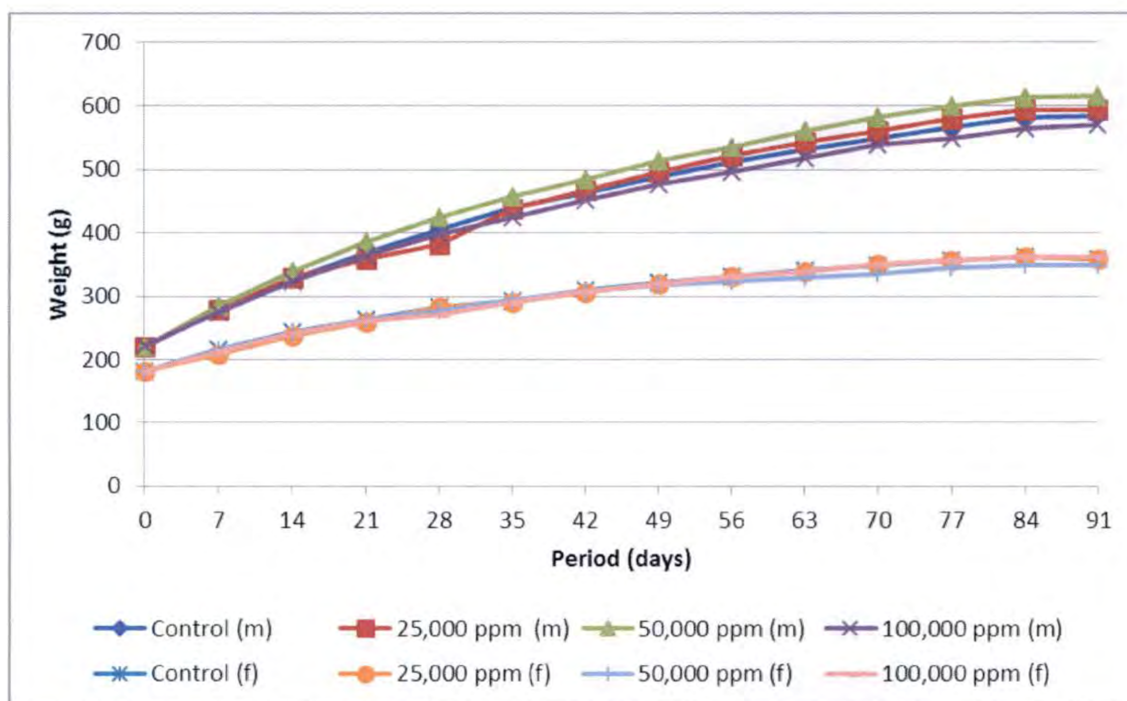

Figure 3. Mean body weights of male and female rats consuming diets containing kokum fat

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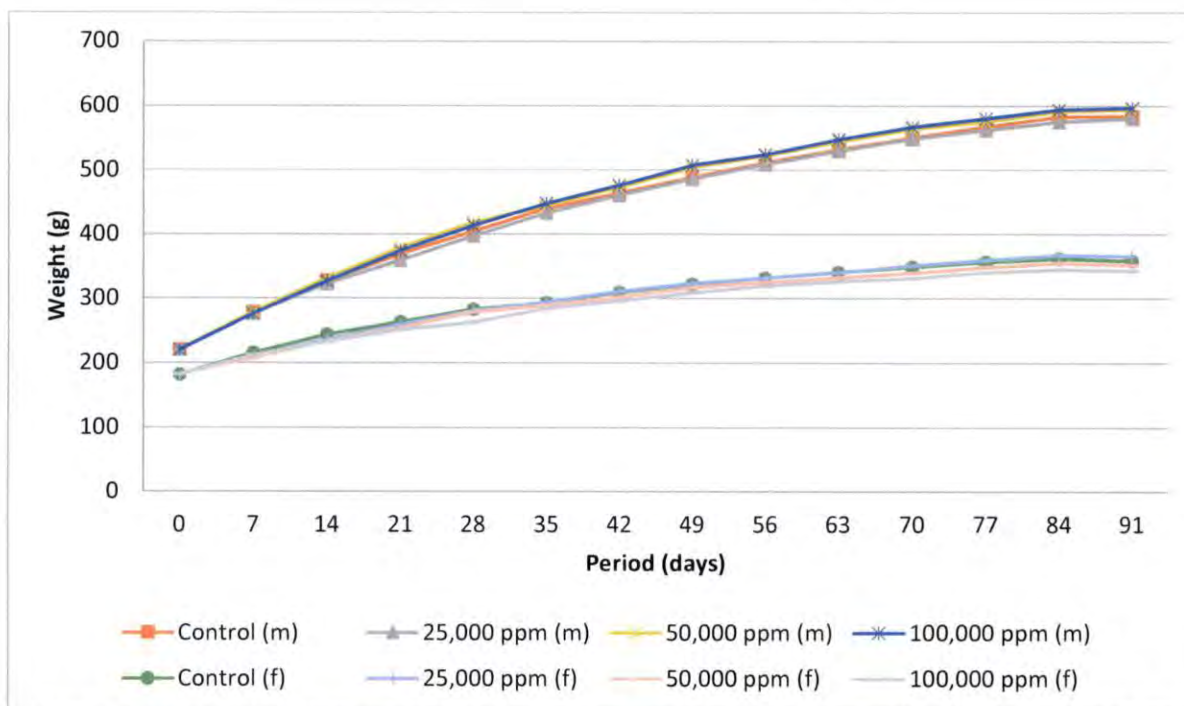

Figure 4. Mean body weights of male and female rats consuming diets containing algal structuring fat

Overall and calculated 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 and mean food efficiency for the male rats consuming the algal structuring fat (data not shown) 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 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 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 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 at the end of the study (Table 16 and Table 17) 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. Hematology changes reported in female rats 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 ($502 – 1651 \times 10^3/\mu L$), 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 not considered toxicologically relevant. There were no statistically significant differences in hematology parameters between the algal structuring fat and kokum fat test groups ($p > 0.05$).

Clinical chemistry values were not different between male or female 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. 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 incidental or sporadic findings are provided in Table 15. 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 18). There were no absolute or relative organ weight changes in either the male or female rats that were adverse (Table 18 and Table 19). 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 19). However, these changes were not associated with histomorphological adrenal findings and were therefore non-adverse in nature. The no-observed-adverse-effect-level (NOAEL) for the algal structuring fat was 100,000 ppm under the conditions of this study, the highest dietary concentration provided, which corresponds to a dietary NOAEL of 5299.2 mg/kg bw/day in male rats and 6318.8 mg/kg bw/day in female rats.

Table 15. Sporadic and incidental findings after feeding algal structuring fat or kokum fat for 13 weeks (Matulka *et al.*, 2016)

Dose Group	Gender	Macroscopic Observation
Control group	Females (7/19)*	Fluid-filled uteri
Kokum fat (25,000 ppm)	Females (6/10)	Fluid-filled uteri
Algal structuring fat (100,000 ppm)	Females (3/10)	Fluid-filled uteri
Kokum fat (25,000 ppm)	Male (1/10)	Milky-filled dermal edema in the ear
Kokum fat (25,000 ppm)	Female (1/10)	Adipose tissue focus, retroperitoneum tan, increased firmness and of irregular shape
Kokum fat (50,000 ppm)	Male (1/10)	Adipose tissue focus, mesentery, round, yellow, firm, increased firmness, 2x10 mm
Algal structuring fat (100,000ppm)	Male (1/10)	Epididymides nodule, right of the tail, yellow, 3x3 mm
Algal structuring fat (25,000ppm)	Male (1/10)	Soft left testes, and small left epididymides
Algal structuring fat (50,000 ppm)	Male (1/10)	Soft left testes and small left epididymides
Algal structuring fat (100,000 ppm)	Male (1/10)	Small testes (bilateral) and small epididymides (bilateral)
Algal structuring fat (100,000 ppm)	Male (1/10)	Enlarged right testes (18x9 mm), small left testes (25x12 mm) and small right epididymides
Kokum fat (100,000 ppm)	Female (1/10)	Pale, diffuse liver with irregular surface, correlated to marked bile duct ectasia; kidney focus (bilateral), tan, multifocal, pinpoint with an irregular bilateral, multifocal, diffuse surface due to cysts in the kidneys
Algal structuring fat (100,000 ppm)	Male (1/10)	Thymus discoloration (multifocal), with redness due to agonal hemorrhage
Algal structuring fat (100,000 ppm)	Male (1/10)	Small left adrenal gland lobe (no histologic correlate)
Kokum fat (25,000 ppm)	Female (1/10)	Liver focus on right lateral lobe (3x6 mm)
Kokum fat (50,000 ppm)	Male (1/10)	Liver focal area indentation on left lateral lobe (7 mm)
Algal structuring fat (50,000 ppm)	Female (1/10)	Lung discoloration (red) on the left lobe at the cranial aspect (1.5x2 cm)

*Number of animals with observation out of total number of animals in group

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Table 16. Hematology, coagulation, and clinical chemistry parameters in male rats following 13 weeks dietary treatment with kokum fat or algal structuring fat

Parameter	0 ppm (n=19)[a]	25,000 ppm KF (n=10)	50,000 ppm KF (n=10)	100,000 ppm KF (n=10)	25,000 ppm AF (n=10)	50,000 ppm AF (n=10)	100,000 ppm AF (n=10)
Hematology							
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							
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							
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	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

Table 16. Hematology, coagulation, and clinical chemistry parameters in male rats following 13 weeks dietary treatment with kokum fat or algal structuring fat

Parameter	0 ppm (n=19)[a]	25,000 ppm KF (n=10)	50,000 ppm KF (n=10)	100,000 ppm KF (n=10)	25,000 ppm AF (n=10)	50,000 ppm AF (n=10)	100,000 ppm AF (n=10)
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							
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#	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#	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#	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#	167±77#	155±90	157±63

[a] Control group.

[*] Statistically significant from Group 1 control ($P < 0.05$); #n = 9; †n = 18; ‡n = 17. ABAS = absolute basophils; AEOS = absolute eosinophils; AF = algal structuring fat; 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 cell.

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Table 17. Hematology, coagulation, and clinical chemistry parameters in female rats following 13 week dietary treatment with kokum fat or algal structuring fat

Parameter	0 ppm [a] (*n*=19)	25,000 ppm KF (*n*=10)	50,000 ppm KF (*n*=10)	100,000 ppm KF (*n*=10)	25,000 ppm AF (*n*=10)	50,000 ppm AF (*n*=10)	100,000 ppm AF (*n*=10)
Hematology							
RBC (10^6/µL)	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/µL)	978±98	1024±101	1027±121	1117±105*	947±77	1033±135	1042±85
WBC (10^3/µL)	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/µL)	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/µL)	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/µL)	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/µL)	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/µL)	0.12±0.05	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/µL)	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/µL)	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							
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							
AST (U/L)	68±17	63±11	69±10	68±28	67±13	63±9#	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#	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

Table 17. Hematology, coagulation, and clinical chemistry parameters in female rats following 13 week dietary treatment with kokum fat or algal structuring fat

Parameter	0 ppm [a] (n=19)	25,000 ppm KF (n=10)	50,000 ppm KF (n=10)	100,000 ppm KF (n=10)	25,000 ppm AF (n=10)	50,000 ppm AF (n=10)	100,000 ppm AF (n=10)
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							
Urine volume (mL)	4.1±3.2†	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‡	6.1±0.3	6.1±0.2	6.3±0.5	6.3±0.4	6.3±0.5	6.2±0.3#
Specific gravity	1.048±0.026‡	1.061±0.029	1.033±0.014	1.040±0.024	1.039±0.024	1.048±0.019	1.033±0.018#
URO (EU/dL)	0.3±0.3‡	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.3	0.3±0.3	0.2±0.0#
UMTP (mg/dL)	98±126†	115±73	43±26	107±153	56±43	73±40	80±125

[a] Control group.

[*] Statistically significant from Group 1 control ($P < 0.05$); #n = 9; †n = 18; ‡n =1 7. ABAS = absolute basophils; AEOS = absolute eosinophils; AF = algal fat; 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.

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Table 18. Summary of mean terminal body and organ weights in male rats following 13 week dietary treatment with kokum fat or algal structuring fat

Parameter	0 ppm[a] (n=19)	25,000 ppm KF (n=10)	50,000 ppm KF (n=10)	100,000 ppm KF (n=10)	25,000 ppm AF (n=10)	50,000 ppm AF (n=10)	100,000 ppm AF (n=10)
Mean terminal body and organ weights							
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	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	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	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	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	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	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	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	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	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*							
Adrenals/TBW	0.1081±0.0225	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	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	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	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	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	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	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	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	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*							
Adrenals/BrW	0.0265±0.0044	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	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	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	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	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	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	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	0.1449±0.0328	0.1496±0.0326	0.1254±0.0364	0.1187±0.0274	0.1210±0.0157	0.1438±0.0416

[a] Control group.

AF = algal structuringfat; BrW = brain weight; KF = kokum fat; TBW = terminal body weight; *Organ-to-body weight ratios and organ-to-brain weight ratio numbers are multiplied by a factor of 1000 for clarity; ** $P < 0.05$; *** $P < 0.01$ Statistically significant from Group 1 control.

Table 19. Summary of mean terminal body and organ weights in female rats following 13 week dietary treatment with kokum or algal structuring fat

Parameter	0 ppm[a] (n=19)	25,000 ppm KF (n=10)	50,000 ppm KF (n=10)	100,000 ppm KF (n=10)	25,000 ppm AF (n=10)	50,000 ppm AF (n=10)	100,000 ppm AF (n=10)
Mean terminal body and organ weights							
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	0.0770±0.0087	0.0712±0.0181	0.0583±0.0147*	0.0711±0.0085	0.0698±0.0095	0.0623±0.0142
Brain (g)	2.126±0.084	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	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	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	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	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	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	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	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							
Adrenals/TBW	0.2106±0.0367	0.2245±0.0230	0.2105±0.0410	0.1683±0.0413*	0.2032±0.0340	0.2082±0.0321	0.1889±0.0404
Brain/TBW	6.221±0.737	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	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	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	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	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	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	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	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							
Adrenals/BrW	0.0344±0.0058	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	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	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	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	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	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	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	0.438±0.147	0.387±0.083	0.355±0.079	0.356±0.068	0.431±0.163	0.357±0.101

[a] Control group.
[b] Treatment with kokum fat.
[c] Treatment with algal structuring fat; *$P < 0.05$ Statistically significant from Group 1 control; AF = algal structuring fat; BrW = brain weight; KF = kokum fat; TBW = total body weight.

A subchronic (19 week) feeding study to evaluate the effect of high stearic acid (SA)-containing fats on markers for *in vivo* lipid peroxidation in weanling female Sprague Dawley rats from the consumption of a basal diet to which either beef tallow (BT), cocoa butter (CB), soybean oil (SO) or menhaden oil (MO) was added to provide fat at eight percent of the diet (Saari Csallany *et al.*, 2005). The fats that contained high levels of stearic acid (as a total of the fatty acid composition) were the beef tallow fat (16% stearic acid) and cocoa butter (33.8% stearic acid). While food intake and body weights were not statistically different between groups ($p > 0.05$), the consumption of the beef tallow diet resulted in lower ($p < 0.05$) levels of urinary total non-polar and polar lipophilic aldehydes and related carbonyl substances (markers of secondary lipid peroxidation products). The cocoa butter-based diet was no different than the soybean or menhaden oil-based diets for these parameters. The serum's resistance to copper-induced oxidation of fatty acids was significantly ($p < 0.05$) higher in the beef tallow- and cocoa butter-based diets, compared to the soybean- and menhaden-based diets. However, the serum ORAC[87] value was significantly ($p < 0.05$) higher in the soybean-fed group, compared to the other groups. The authors concluded that "high SA containing BT diet is responsible for (a) lower serum oxidation and (b) lower *in vivo* lipid oxidation measured by the excretion of the urinary secondary oxidation products and (c) no increase in triglyceride and serum cholesterol levels when compared to SO diet" (Saari Csallany *et al.*, 2005).

In summary, although statistically significant values were reported for several endpoints in the subchronic study conducted on algal structuring fat, none of the changes were considered toxicologically relevant to the consumption of the algal structuring fat at the mean intake of 1285.6, 2594.3 and 5299.2 mg/kg bw/day in male rats and 1606.0, 3069.7 and 6313.8 mg/kg bw/day in female rats, as they were within historical control values, were not dose-dependent in nature, and were not accompanied by corresponding clinical or histopathological changes. The NOAEL for this subchronic study of dietary administration of algal structured fat to rats was 100,000 ppm, the highest dose tested, corresponding to 5299 mg/kg bw/day in male rats and 6313 mg/kg bw/day in female rats. Other studies conducted in rats with stearic acid-rich fats found that consumption at levels of 8 – 10% of the diet also did not result in toxicity. Overall, the subchronic studies show that the algal structuring fat or diets containing high levels of stearic acid do not result in toxicity under the conditions of the study parameters.

6.15. Genotoxicity Studies

Algal structuring fat was analyzed for the ability to produce genotoxicity, as assayed for mutagenic potential *in vitro* in the reverse bacterial mutation assay, as well as *in vivo* in a mouse model for the potential to produce chromosomal aberrations (Matulka *et al.*, 2016), as discussed in the following sections. In addition, a stearic acid-rich structured TAG-based fat (*i.e.*, Salatrim®) was previously evaluated for the potential to promote genetic damage, as assayed in the *in vitro* mammalian chromosomal aberration assay, the unscheduled DNA synthesis (UDS) assay and the hypoxanthine-guanine phosphoribosyltransferase (HPRT) mammalian cell mutagenesis assays, as well as the *in vivo* bone marrow micronucleus assay conducted in rats (Hayes *et al.*, 1994a). The structured fat utilized in these studies was predominantly composed of stearic acid ($\geq$ 50%) and lesser amounts of other saturated fatty acids, mainly acetic (21.1%), palmitic (2.37%) and

[87] ORAC = Oxygen Radical Absorbance Capacity

propionic acids (2.58%), while having less than 1% of any of the mono- or poly-unsaturated fatty acids.

6.15.1. Bacterial Reverse Mutation Assay

The potential for the algal structuring fat to induce gene mutations in bacteria was evaluated using the bacterial reverse mutation test (Matulka *et al.*, 2016). The bacterial reverse mutation assay, under the OECD guidelines, uses amino acid-requiring strains of *Salmonella typhimurium* and *Escherichia 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 for Experiment I. Experiment II utilized eight dose levels (40, 80, 160, 320, 640, 1280, 2560 and 5000 μg/plate). Tester strain concentrations of approximately 1×10^9 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 time 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.

No toxic effects of 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 (Mortelmans and Zeiger, 2000; Gatehouse, 2012). 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 and therefore, each phase of the test was considered valid.

Algal structuring fat is solid at room temperature. The test substance temperature was maintained at 45°C during preparation to allow 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 the 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. There were no concentration-related or substantial test substance-related increases in the number of revertant colonies 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). Under the conditions of the study, 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.

6.15.2. Chromosomal Aberration Assay

The mammalian *in vivo* chromosome aberration assay was used to assess the ability of 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 2000 mg/kg bw (orally) in a preliminary range-finding toxicity experiment. Based on the OECD guidelines, three oral dose 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 hours and 48 hours 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 48 hours 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 hours after test oil/negative control administration, and 24 hours after treatment of the positive control group (*n*=five/sex). The bone marrow cells were fixed, stained and examined microscopically. Cytogenetic damage was assessed by scoring 100 metaphases/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.

Exposure to a single oral dose of algal structuring fat for 24 or 48 hours did not affect the mean number of chromosomal aberrations in the bone marrow of the mice of either sex (Table 20). The mean values noted for algal structuring fat dose levels of 400, 1000 and 2000 mg/kg bw in the mice 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 not significantly different between control and treatment groups, but the index was significantly ($p < 0.05$) reduced in the positive control mice, confirming the ability of this test to reveal cytotoxicity. Male mice exposed to 2000 mg/kg bw for 24 hours showed mean aberrant cell values of 0.2% and female mice exposed for either 24 or 48 hours exhibited no aberrant cells (0.0%). There was no dose-dependent, biologically relevant increase in chromosomal aberrations following treatment with algal structuring fat. 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 algal structuring fat-dosed animals.

Table 20. 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 (400 mg/kg bw), 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 (1000 mg/kg bw), 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 (2000 mg/kg bw), 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 (2000 mg/kg bw)#, 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

[a] Aberrant cells, excluding chromosomal gaps.
[b] Five mice (50 metaphases); [c] Four mice (50 metaphases), one mouse (100 metaphases).
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; #n=7

In an *in vitro* chromosomal aberration assay conducted in Chinese Hamster Ovary (CHO) cells, the Salatrim® fat was soluble in the culture medium at a concentration up to 40 µg/mL, but

became a suspension at higher concentrations. In the preliminary/cytotoxicity assay, no significant cell cycle delay or reduced mitotic index was seen, and therefore a dose range of 0, 250, 500 and 1000 μg/mL Salatrim® was used in the definitive assay. There were no major changes in the mitotic index in the CHO cells exposed to Salatrim®, nor were there changes in the percent of cells with abnormal chromosomes, chromatid deletions/exchanges, or in the number of structural aberrations, either in the presence or absence of metabolic activation (Hayes *et al.*, 1994a). Cocoa butter, which is similar in fatty acid composition to algal structuring fat, was also evaluated for the ability to increase sister chromatid exchanges (SCE) *in vitro* in Chinese hamster bone marrow cells (Renner and Münzner, 1982). The test was performed with 5-bromo-deoxyuridine (BrdU) tablets that were subcutaneously implanted into Chinese hamsters for a 26 hour treatment time, and 50 metaphases analyzed/animal and four animals analyzed. The cocoa butter was administered to the Chinese hamsters in 0.2 g increments at 90 minute intervals for a total of 0.6 g. The cocoa butter did not cause an alteration of the number of SCEs (Renner and Münzner, 1982).

6.15.3. Unscheduled DNA synthesis (UDS) study

In a UDS study, Fischer 344 rat hepatocytes were isolated and approximately 300,000 viable cells/mL were cultured with the Salatrim® fat at concentrations of 0 – 1000 μg/mL for 19 hours at 37°C (the high dose was limited by insolubility of the fat). After exposure, the cultures were washed, fixed, and stained with 1% methyl-green pyronine Y. Quantitative autoradiographic grain counting was performed on at least 30 morphologically unaltered cells in a randomly selected field. The *percentage* of cells in repair indicated the response throughout the liver culture. For each experiment (the UDS assay was repeated twice), 90 cells *per* dose were scored. A test material was determined positive if the mean number of grains *per* nucleus was greater than five, and determined negative if the net grains *per* nucleus was less than zero and the *percentage* of cells in repair (% IR) was < 10% for all dose groups (Hayes *et al.*, 1994a).

The UDS for Salatrim® was not increased when compared with corn oil or the solvent control, while the positive control 2-(acetylamino)fluorine (2-AAF) increased the net number of nuclear grain counts (the main parameter of DNA synthesis in this assay) and the *percentage* of hepatocytes in DNA repair. The authors stated that, according to the criteria for this study, the Salatrim® fat did not induce UDS in isolated rat hepatocytes (Hayes *et al.*, 1994a).

6.15.4. HPRT Mammalian Cell Gene Mutation Assay

The stearic acid-rich Salatrim® fat was evaluated for genetic mutations in mammalian CHO cells, with or without metabolic activation (S9 preparations). The Salatrim® fat was added to cultured CHO cells at six concentrations ranging from 31.25 – 1000 μg/mL for both the preliminary assays to determine cytotoxicity and dose range, and for the definitive study, which was conducted in duplicate using duplicate cultures for each replicate. Cytotoxicity was determined by cell detachment concentration from the culture flask and cloning efficiency (CE) relative to the solvent control. Analyzing the phenotypic expression of induced mutants led to the determination of the mutant frequency (*i.e.*, the ratio of mutant cells to nonmutant cells), which was calculated by dividing the number of resistant (to 6-thioguanine) colonies by the number of unselected viable colonies. The results were considered positive if the number of mutant colonies increased in a dose-related fashion and the mutant frequencies of duplicate cultures were at least three times the average of the solvent-treated control cultures. The authors stated that "the Salatrim® fat presented no evidence of mutagenic potential under the conditions of the assay",

while the positive controls (ethyl methanesulfonate and 3-methylcholanthrene) increased the mutant frequency to greater than three-times the control frequency, validating the assay (Hayes *et al.*, 1994a).

6.15.5. *In vivo* Rat Bone Marrow Micronucleus Assay

Bone marrow was obtained from rats that were part of a 13-week subchronic toxicity study in which Crl:CD BR VAF/Plus rats (n=18-20/sex/dose) were exposed to the Salatrim® fat at 20% of the diet for 13 weeks, and compared to a corn oil-fed control group. Duplicate bone marrow slides were prepared at necropsy, with one set fixed in methanol, stained with acridine orange and analyzed using fluorescent microscopy. Polychromatic erythrocytes (n=1,000/rat) were scored for micronuclei, with the frequency of micronucleated cells expressed as *percent* micronucleated cells, based upon the total number of scored polychromatic erythrocytes (typical micronucleated erythrocyte frequency for this strain of rat was stated by the author to be 0.0 – 0.4%). Although a positive control group was not included in this assay (as the bone marrow was taken from animals in a 13-week dietary study), the incidence of micronucleated polychromatic erythrocytes from rats that consumed Salatrim® fat at 10% of the diet was no different from the control rat group that consumed corn oil at 10% of the diet. The authors concluded that the Salatrim® fat was negative for the ability to increase micronuclei formation in the bone marrow of rats (Hayes *et al.*, 1994a).

6.15.6. Genotoxicity Conclusion

The algal structuring fat was evaluated for genotoxic potential by analyzing for mutagenic potential in the *in vitro* bacterial reverse mutation assay and for the potential to produce chromosomal aberrations *via* the *in vivo* mouse chromosomal aberration assay. Concentrations up to 5,000 µg/mL algal structuring fat did not increase the number of mutations in the bacterial strains *S. typhimurium* TA98, TA100, TA1535 and TA1537 and *E. coli* WP2 *uvrA*. In the *in vivo* mouse micronucleus assay, administration of algal structuring fat *via* gavage at up to 2000 mg/kg bw did not affect the mean number of chromosomal aberrations in the bone marrow of the mice of either sex. Salatrim®, a stearic acid-rich structured fat, was also evaluated for genotoxicity, and was found to lack genotoxic potential when evaluated *in vitro* for unscheduled DNA synthesis, the formation of chromosomal aberrations and the ability to mutate mammalian cell DNA, and did not increase the formation of micronuclei in bone marrow of rats that consumed Salatrim® at up to 10% of the diet for 13 weeks. Overall, the data confirm that the algal structuring fat, a stearic acid-rich fat, is not genotoxic under the conditions of accepted methods used to determine the genetic potential of food substances, and that a stearic acid-rich food ingredient does not increase the potential for genotoxicity.

6.16. Corroborative Pathogenicity Study in *P. moriformis* S2014

In support of the non-pathogenic nature of the *P. moriformis* S7737 source organism for algal structuring fat, a pathogenicity study using the rat model was conducted on *P. moriformis* S2014, a closely related strain that was also produced from the wild-type strain *P. moriformis* S376 (categorized as a BSL 1 microorganism in the ATCC[8] but BSL 2 in the SAG culture collection[9]), through classical mutagenesis and genetic engineering (Solazyme, 2012). The study was designed to determine if microbial agents would become systemic in the body and survive or propagate after oral administration. The in-life dosing and tissue collection portion of the study was based on the

U.S. EPA Health Effects Test Guidelines, OPPTS[88] 885.3050, Acute Oral Toxicity/Pathogenicity Study (1996) and U.S. FDA Toxicological Principles of the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 3a: *Short-Term Toxicity Studies with Rodents* (2003), and was conducted under good laboratory practice (GLP) with one exception.[89] The tissue culture and analysis portion of the study was also conducted under GLP[90] in accordance with OPPTS 885.3050.

Rats (Crl:SD CD IGS strain, 15 – 16 weeks of age) were randomly assigned to either treatment (*n*=twelve/sex) or vehicle control (*n*=four/sex) group. The live microalgal cells (~$2.5x10^8$ CFU[91]/rat) in 2 mL vehicle (Defined EBO2[92] minus Co^{2+} and nitrogen) was administered *via* oral gavage to each rat as a single dose in the treatment group, and the vehicle alone was provided to each rat in the vehicle group. All the treatment rats and one-half the vehicle control rats (two males, two females) were housed in a separate room. All rats were monitored for adverse signs twice daily with cage-side observations twice the first day and once *per* day thereafter, with 2016CM Harlan Teklad Global Rodent Diet® (Harlan Teklad, Inc., Indianapolis, IN) and filtered tap water provided *ad libitum*. Body weights (controls) were obtained during acclimation and on Days 1, 3, 7, 14, and 21, with terminal weights recorded on Day 22. Treatment rat body weights were obtained during acclimation and on Day 1, with terminal weights recorded for the first subgroup (*n*=three/sex) on Day 4. For the second subgroup (*n*=three/sex), body weights were recorded on Day 7 with terminal weights recorded on Day 8. Body weights for the third subgroup (*n*=three/sex) were recorded on Days 7 and 14, with terminal weights on Day 15. The final (fourth) subgroup (*n*=three/sex) bodyweights were recorded on Days 7, 14, and 21; terminal body weights were taken on Day 22. Tissue[93], K_2EDTA[94]-treated blood and plasma, and fecal samples were collected over the three-week period post-dose and transferred (at ~4°C) from PSL (Dayton, NJ) to BSL Bioservice (Planegg, Germany) for analysis.

No mortalities occurred during the in-life part of the study, and no effect on mean body weight, mean body weight gain or clinical signs were reported for the treatment groups, when compared to the control groups. No differences between treatment and control groups were identified in any evaluated parameter. No macroscopic differences were observed at necropsy between the groups, other than an incidental finding of slight alopecia of the hind limb of one treatment group male. Blood, tissue and fecal samples were analyzed along with negative and positive controls for the presence of *P. moriformis* cells, utilizing a surface spread method with plate counting for CFU/plate of the microalgae. The sample and control plates were prepared in triplicate, and each plate was analyzed in duplicate. The positive control plates were comprised of 2 – 7 day old *P. moriformis* S2014 cultures plated at 1:100, 1:1000, 1:10,000 and 1:100,000

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[88] OPPTS=Office of Prevention, Pesticides and Toxic Substances.

[89] Dose formulation (identity and concentration verification) was the responsibility of the study sponsor, Solazyme Inc. (Solazyme, 2012).

[90] Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to §19a as amended and promulagated on July 2, 2008 (BGBl. I Nr. 28 S. 1146) and OECD Principles of Good Laboratory Practice (as revised in 1997), in the EOCD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring – Number 1. Environment Directorate (1998).

[91] CFU=colony forming unit.

[92] Defined EB02 = A proprietary media formulation developed by Solazyme containing:
[ingredients that include; sodium and potassium phosphate, citric acid, sulfates, metals, minerals and mineral salts, vitamins and glucose]

[93] Brain, lung, liver, spleen, kidney and lymph nodes.

[94] K_2EDTA=Dipotassium ethylenediaminetetraacetic acid.

dilutions. Plate counting determined that algal colony counts were below the limits of detection for all treatment and control tissue, blood, plasma and feces samples for all collection days. No presence or growth of *P. moriformis* was observed in the negative control plates (< 1 CFU/mL negative control inoculum, equivalent to < 1 CFU/plate). *P. moriformis* was observed in the positive control plates and 2-4 day cultures, with the mean cell count in the positive controls (n=5) at 7.26×10^7 CFU/mL positive control inoculum, equivalent to 7.26×10^7 CFU/plate. The mean count (n=5) in the 2-4 day cultures was 1.27×10^8 CFU/mL.

No adverse effects related to the treatment with live *P. moriformis* S2014 were found in any rat, and no viable counts were detected in any examined tissue or fluid samples collected during the three-week period following administration of the microalgal culture. Under the conditions of the study, *P. moriformis* S2014 was not toxigenic or pathogenic in this unpublished study (Solazyme, 2012).

6.17. Reproductive study

A three-generation nutritional and toxicological evaluation was conducted in Wistar rats on mango kernel oil (MKO), a fat containing 41.1% stearic acid and 43% oleic acid as a *percentage* of total fatty acid content (Rukmini and Vijayaraghavan, 1984). The rats (n=15/sex/group) were fed a diet containing either mango kernel oil at 10% of the diet, or 10% of the diet as ground nut (*i.e.*, peanut) oil (GNO), for 22 weeks. Weekly body weights and food intake were assessed, and breeding studies were conducted such that all rats consumed the MKO diet for 22 weeks. At the end of the 22 week period, the liver and serum total lipids, total cholesterol and triglycerides were estimated. At necropsy, organ weights of the livers, spleens, lungs, hearts and testes or ovaries at each point of sacrifice were determined, stated as a percentage of the bodyweight, then the tissues were fixed and sections stained with hematoxylin and eosin and examined by light microscopy. Reproductive performance was assessed through the following parameters: *percentage* of conception, mean litter size, mean birth weight, mean weanling weight, preweanling mortality and mean number of days taken to deliver from the date of mating.

The feed-efficiency ratio of the MKO-fed animals compared favorably with the GNO-fed groups, over the three generations. The reproductive performance parameters evaluated in this study (stated above) did not differ between the two test diets. Serum and liver total cholesterol, total lipid and triglyceride levels were similar between dose groups over the whole three generation period. In addition, there were no statistically significant differences in organ weights or histopathological analysis of any of the tissues between the two groups during this three generation study. This study shows that a stearic acid/oleic acid-rich fat similar to algal structuring fat in stearic acid content, when consumed at 10% of the diet for 22 weeks, did not cause toxicological or reproductive adverse effects in rats (Rukmini and Vijayaraghavan, 1984).

6.18. Carcinogenesis

No studies evaluating potential carcinogenicity of *P. moriformis* or any *Chlorella* or *Prototheca* species were found in the scientific literature.

6.19. Observations in Humans

Saturated fatty acid consumption in general has previously been associated with an increased risk of coronary heart disease (CHD), with the U.S. dietary guidelines advising to reduce the consumption of saturated fatty acids by replacement with polyunsaturated fatty acids (Dietary

Guidelines Advisory Committee, 2015). A recent prospective cohort study among 91,981 women reported that saturated fatty acid consumption is positively associated with a greater risk of sudden cardiac death, which appeared to the authors to be "mediated through the development of interim CHD and predisposing diseases" (Chiuve *et al.*, 2012). However, the consumption of stearic acid most likely does not contribute to this risk (Grundy and Denke, 1990). Hunter *et al.* (2001) conducted a randomly controlled, cross-over study to investigate the effects of stearic acid-, oleic acid- and linoleic acid-rich meals on postprandial hemostasis, as changes in hemostasis were previously associated with an increased risk in CHD. The diets provided 38% of the total daily energy intake as fat, and of that, the stearic acid-rich diet contained 34.1% stearic acid and 36.6% oleic acid as the fatty acid content. Male volunteers (n=6) randomly consumed diets rich in stearic acid, oleic acid or linoleic acid for 14 days, then plasma lipids and hematological parameters (Factor VIIc (FVIIc), Factor VIIa (FVIIa), FVII antigen, plasma triacylglycerol, C-reactive protein (CRP)) and blood chylomicrons were measured. The authors found that responses were due to the consumption of a fatty meal, as opposed to any specific fatty acid, with the consumption of meals containing a physiological fat load by healthy young individuals inducing potentially pro-coagulant alterations in the hemostatic system. In addition, these changes were independent of the fatty acid composition of the test meal, being evident when meals were rich in stearic, oleic or linoleic acids (Chiuve *et al.*, 2012).

Sanders *et al.* (2003) found that consumption from a single test meal of a symmetrical stearic acid-rich triacylglycerol with oleic acid in the *sn*-2 position (as is found in cocoa butter) by male subjects (n=17) in a randomized crossover trial that provided 16.1 g stearic acid (and 11.5 g oleic acid), was absorbed more rapidly from the gastrointestinal tract than an asymmetrical triacylglycerol (synthetic triacylglycerol) with long-chain saturated fatty acids in the *sn*-2 position (containing 37.9 g oleic acid and 1.8 g stearic acid), with the symmetrical stearic fatty acid consumption leading to an increase in FVIIa. However, there were no significant differences in the plasma levels of total and HDL-cholesterol (HDL-C) between the two different stearic acid-rich products, or between fasting and consumption of the test meals (Sanders and Berry, 2005). This work built upon an earlier study in which Sanders *et al.* (2001) had reported that consumption of a meal containing either stearic acid (approximately 19 g) (from cocoa butter) or oleate (from sunflower oil) significantly ($p < 0.05$) increased FVIIc, while a meal containing a structured triacylglycerol (Salatrim) at similar stearic acid amount did not increase this parameter, while all three test meals increased tissue plasminogen activator activity and decreased plasminogen activator type 1 (PAI-1) [95] activity ($p < 0.05$).

A clinical study (randomized cross-over dietary intervention) was performed to evaluate if a stearic acid-rich diet (when compared to a palmitic acid rich diet) consumed for four weeks by men (n=13) would adversely alter hemostatic factors or platelet morphology, fatty acid profile, or platelet aggregation (Kelly *et al.*, 2001). Consumption of the stearic acid-rich diet (providing 19 g stearic acid/day) significantly ($p < 0.05$) increased the fraction of stearic acid in the platelet phospholipids, as would be expected, while significantly ($p < 0.05$) decreasing mean platelet volume, coagulation factor VII activity and plasma lipid concentrations, when compared with the baseline (*i.e.*, prior to test diet consumption) values. The authors concluded that "results from this study indicate that stearic acid (19 g/day) in the diet has beneficial effects on thrombogenic and atherogenic risk factors in males" (Kelly *et al.*, 2001).

[95] PAI-1 activity = Tissue plasminogen activator = a serine protease that cleaves pro-enzyme plasminogen into active plasmin, involved in the breakdown of blood clots.

Consumption of a stearic acid-rich meal (two meals composed of 42% stearic acid, consumed twelve hours apart) by men (*n*=10, ages 21 – 28 years) with no history of atherosclerotic disease resulted in an increase in plasma total triacylglycerol and fibrinolytic activity, with a decrease in PAI-1 activity and t-PA-PAI-1 complex[96], which was no different from individuals consuming myristic acid and may be due to the basic change to consumption of a fat-rich meal. The stearic acid-rich meal provided a consistent 1.2 g total fat/kg body weight of subject, resulting in a stearic acid consumption ranging from 34.5 – 51 g/subject. The authors concluded that consumption of fats high in stearic acid did not lead to a prothrombotic effect (Tholstrup *et al.*, 1996).

Tholstrop later published results from a single blind cross-over, randomized, postprandial study in women (*n*=10) that compared the effects of a fat load of cocoa butter to olive oil intake and found that there was no difference between the diets on serum TAG, hsCRP and IL-6[97] concentrations and, no association between inflammatory markers and postprandial lipemia.[98] The authors concluded that "fat loads with cocoa butter high in stearic acid did not increase hsCRP and IL-6 compared to olive oil postprandially" (Tholstrup *et al.*, 2011). Supporting this conclusion that a stearic acid-rich diet does not increase postprandial lipemia was a review by Sanders and Berry (2005) of the clinical data they published on the influence of stearic acid on postprandial lipemia and hemostatic function, in which the authors concluded that "taken together, these data refute the hypothesis that stearic acid-rich fats have adverse effects on postprandial lipemia, fibrinolytic, and FVIIc activities." Conversely, Karupaiah *et al.* (2011) found that consumption of a daily diet containing 25.76% palmitic acid and 33.37% stearic acid (as *percentages* of the total fat, which resulted in consumption of 12.9 g palmitic acid and 16.7 g stearic acid from the daily diet) for seven days significantly increased plasma TAG and decreased HDL-C ($p < 0.05$), when compared with a similar diet containing palmitic acid or a combination of lauric (C12:0) and myristic (C14:0) fatty acids, with fat clearance being slowest from the stearic acid-rich diet, based on the lipemia analysis. The authors suggested that a slower fat clearance may be conducive to a prothrombotic state and an increase in PAI-1, but this was not the case in the Tholstrup *et al.* (1996) study, discussed above. The authors only concluded that "the nature of dietary saturates clearly influenced fat digestion and absorption in the subjects" (Karupaiah *et al.*, 2011).

Storm *et al.* (1997) reported that, based on a randomized, crossover study conducted in men (*n*=8) and women (*n*=7) who consumed 30 g of either stearic acid or palmitic acid daily for three weeks, "stearic acid- and carbohydrate-rich diets induced similar changes in lipid profiles in NIDDM[99] subjects, eliciting a significantly lower total cholesterol than the palmitic acid-rich diet." Snook *et al.* (1999) also found that a stearic acid-rich diet (a diet providing approximately 28 g stearic acid/day consumed for five weeks by 18 women) in which the majority of the stearic acid came from a synthetically produced tristearin significantly decreased high density lipoprotein cholesterol-3 (HDL_3), compared to the pre-consumption period. In addition, the stearic acid consumption significantly decreased the rate of cholesterol esterification and cholesterol ester transfer ($p < 0.05$). The authors concluded that consumption of a stearic acid-rich diet did not have an adverse effect on the concentrations and metabolism of serum lipoproteins, being "neutral" in

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[96] t-PA-PAI-1 complex = biochemical marker for myocardial reinfarction (Wiman *et al.*, 2000).
[97] hsCRP = High sensitivity, C-reactive protein, a marker for cardiovascular disease; IL-6 = Interleukin-6, produced during inflammation.
[98] Lipemia = Turbidity of a blood sample caused by accumulation of lipoprotein particles, with chylomicrons (sample size of 70 – 1000 nm) having the greatest potential in causing turbidity of the sample (Nikolac, 2014).
[99] NIDDM = non–insulin-dependent diabetes mellitus.

cholesterolemic effects on the human body, compared to diets rich in myristic or palmitic acids (Snook *et al.*, 1999).

Compared with fats high in palmitic and myristic-lauric acids, consumption of shea butter (high in stearic acid and similar in composition to algal structuring fat) that provided 30 – 38 g stearic acid for three weeks decreased fasting FVIIc and was not more thrombogenic than unsaturated fatty acids in the postprandial state or other saturated fatty acids (Tholstrup, 2005). Similarly, healthy men (n=50) who were part of a randomized crossover study in which eight *percent* of the dietary energy came from stearic acid (43.9% of the fatty acids as stearic acid, equivalent to approximately 37.8 g stearic acid/day) for five weeks, did not differ in hemostatic risk factors, defined in this study as plasma concentrations of FVIIc or PAP[100] and PAI-1, when compared to a similar oleic acid-rich or carbohydrate-based diet (Gebauer *et al.*, 2014). As indicated above, Factor VII is a primary actor in thrombus formation, and PAI-1 plays a primary role in fibrinolysis, while PAP is a marker for the activation of the fibrinolytic system. Therefore, the Gebauer *et al.* study reports that consumption of stearic acid-rich diets do not contribute to cardiovascular disease risk. In addition, Mensink (2005) evaluated the literature concerning the effects of stearic acid on plasma lipid and lipoproteins in humans, concluding that "effects of stearic acid on LDL cholesterol levels are more comparable to those of oleic acid than to those of the cholesterol-raising saturated FA or *trans* monounsaturated FA."

6.19.1. Allergenicity Potential

A search of the literature did not find any association of allergy or allergic response to *P. moriformis*, any *Chlorella* or *Prototheca* species, or any substance derived from *P. moriformis*. It is highly unlikely that an allergenic response would occur in a person consuming a food containing the algal structuring fat, as the fat is refined, bleached and deodorized after extraction from the *P. moriformis* microalgae. The allergenicity potential of refined vegetable oils has been evaluated, with estimated protein contents of refined oils at approximately 1 – 3 µg, as the refining process removes almost all of the proteins from vegetable oils that have gone through the refining process (Crevel *et al.*, 2000). The authors concluded that in general, vegetable oils "presents no risk of provoking allergic reactions in the overwhelming majority of susceptible people" (Crevel *et al.*, 2000).

6.19.2. Infection Potential

Algal infections are rare in humans and other mammals, even though microalgae are ubiquitous organisms and have been isolated from many food items and drinking water (Jagielski and Lagneau, 2007). Annually, only 2 – 5 human microalgal infections are reported globally, with a total of 160 cases of human protothecosis reported from the first diagnosis of human protothecosis (1964) until 2012 (Todd *et al.*, 2012). *Prototheca* species that are recognized to be opportunistic disease agents are *P. wickerhamii* (the etiological cause of most human protothecosis), *P. zopfii* (the typical causative agent in animals), and *P. blaschkeae* (sometimes confirmed in human or animal infections) (Chandler *et al.*, 1978; Jagielski and Lagneau, 2007; Marques *et al.*, 2008; Mohd Tap *et al.*, 2012).

Protothecosis is typically localized (*i.e.*, cutaneous or articular) or is a disseminated systemic infection (Mohd Tap *et al.*, 2012). Clinical protothecosis is presented as bovine mastitis

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[100] Plasmin-a2-antiplasmin complex.

in cows, while in dogs infection can be severe and disseminated, involving the internal organs, eyes and brain (Jagielski and Lagneau, 2007; Satoh *et al.*, 2010). In humans, protothecosis is typically presented as skin infection, usually from contaminated wounds (Torres *et al.*, 2003). Systemic infection nearly always occurs in individuals that are debilitated with underlying disease or are immunocompromised (Torres *et al.*, 2003; Jagielski and Lagneau, 2007; Ramírez-Romero *et al.*, 2010; Satoh *et al.*, 2010; Mohd Tap *et al.*, 2012). The causative agent in approximately 72% of the human infections was identified as *P. wickerhamii* and in 7% of the cases as *P. zopfii*, with one case of onychomycosis due to *P. blaschkeae* (Marques *et al.*, 2008; Todd *et al.*, 2012). Even strains of *Prototheca* that are recognized to be pathogenic exhibit only low virulence (Lass-Florl and Mayr, 2007).

In summary, *P. moriformis* is not recognized in the scientific literature to be associated with pathogenicity, and although *Prototheca* are ubiquitous in nature, as a species they are of such a low virulence only 160 cases of human protothecosis have been reported over a nearly 50-year period with 11 reported fatalities, and none of which were attributed to *P. moriformis*. It is highly unlikely that the refined, bleached, and deodorized algal structuring fat from a modified strain of *P. moriformis* would cause an infection, even in the most putatively susceptible population.

6.20. Evaluation

Algal structuring fat is a pale yellow to wheat yellow-colored, refined, bleached and deodorized fat containing ≥ 50% stearic acid and ≥ 30% oleic acid (as a percentage of total fatty acid content) which is isolated from classically and genetically modified *Prototheca moriformis*. The algal structuring fat can be used as a partial replacement for saturated dietary fats in a variety of foods, including baked goods, confectionaries, margarine/butter-type products and chocolate-containing products.

The algal structuring fat is stable at ambient temperatures (22 – 27°C) and at 40°C for up to six months, with or without addition of 200 ppm of the antioxidant tertiary butylhydroquinone, and contains minimal levels of heavy metals, pheophorbides and microbials.

The algal structuring fat was well tolerated in rats in a 13-week subchronic dietary study with no observed test substance-related toxicity. Although statistically significant values were reported for some endpoints, these effects did not follow a dose-dependent relationship, and were not toxicologically or clinically relevant. Under the conditions of the study, the NOAEL for algal structuring fat in the diet was 100,000 ppm (10% of the diet), the highest dietary concentration evaluated, corresponding to a dietary NOAEL of 5299 mg/kg bw/day in male rats and 6313 mg/kg bw/day in female rats.

Several repeated-dose studies have been conducted in rats evaluating the safety of stearic acid-rich ingredients or from ingredients produced from different strains of *P. moriformis*. The NOAEL for a ground yellow high-lipid biomass material when administered in the diet for four weeks was 100,000 ppm, the highest dose provided, which corresponded to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats. Several repeat-dose studies evaluated the safety of stearic acid-rich fats, finding that diets containing greater than 30% stearic acid resulted in essential fatty acid deficiencies leading to reduced body weight gain and eventual mortality, but administration of the essential fatty acids restored growth. Evaluation of a structured triacylglycerol composed primarily of stearic acid and acetic acid in a 13-week dietary toxicity

study fed to rats at 10% of the diet found that the structured fat did not elicit toxicologically effects at up to 6400 mg/kg bw/day in the male rats and 7300 mg/kg bw/day in the female rats.

The results of a bacterial reverse mutation and *in vivo* bone marrow chromosome aberration assay show that the algal structuring fat is not mutagenic and not clastogenic. A closely related *P. moriformis* strain (that was classically and genetically modified from the same wild-type strain as the *P. moriformis* strain used to produce algal structuring fat) was evaluated for potential pathogenicity in a study in which rats were acutely dosed with live *P. moriformis* S2014 at approximately 2.5×10^8 CFU/rat, with no viable counts detected in any of the examined tissues. Under the conditions of this unpublished study, the closely related *P. moriformis* strain S2014 was not pathogenic.

It is extremely unlikely that human infection of *P. moriformis* would occur from food containing the refined, bleached algal structuring fat, and no case of human protothecosis has identified any strain of *P. moriformis* as a causative agent of infection, with any protothecosis infections attributed to strains of *P. wickerhamii*, *P. zopfii* and *P. blaschkeae*, but no strains of *P. moriformis*. In addition, it is highly unlikely that an allergenic response would incur from consumption of a food containing the algal structuring fat, as the fat is refined, bleached and deodorized after extraction from the microbial cells and does not contain detectable concentrations of Pheide A, chlorophyll breakdown products or any of the common algal toxins or cyanotoxins. Stearic acid, a saturated fatty acid, was once believed to contribute to coronary heart disease, has been determined in general through both preclinical and clinical trials to be neutral in its effect on parameters associated with increases in coronary heart disease (*e.g.*, lipemia, plasma cholesterol levels, thrombogenesis).

The NOAEL for the algal structuring fat in rats in a 13-week study is 5299 mg/kg/day (equivalent to 317.94 g/day in a 60 kg human). Consumption of stearic acid at up to 37 g/day for five weeks by human subjects in a randomized crossover study did not result in any adverse effects.

The estimated daily intake of algal structuring fat is based on the 90th percentile consumption level of the algal structuring fat from foods supplemented with the algal structuring fat, and is 9.10 g/day. Assuming an average human body weight of 60 kg, this consumption level corresponds to 152 mg/kg bw/day, which is well below the NOAELs observed in animal studies. Thus, the potential theoretical algal structuring fat consumption at the 90th percentile may reach 9.10 g/day. This theoretical intake level represents a conservative estimate because it is unlikely that an individual would consume the algal structuring fat from conventional foods at the 90th percentile level and all selected food products would contain the algal structuring fat.

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7. List of Supporting Data

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